DOCTOR OF SCIENCE (DSC)

Collected publications, 1982-1997

Threadgill, Michael David

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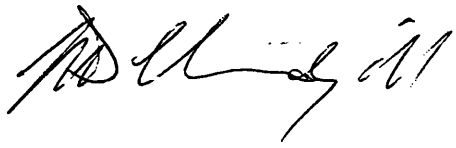

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DECLARATION

I. I am the author of the text which prefaces this compilation of my published work. Each of the works was written by me as sole author, principal author or co-author, as indicated in the preface and on the works.

II. The work described in the published papers has been carried out by me alone, by workers under my direction or by other scientists as parts of collaborations. The involvement of other workers is noted in the preface and in the individual papers. For each of the published papers, I made a major intellectual contribution, as indicated in the preface.

III. No part of this work has previously been submitted by me for the award of a higher degree.

Submission for the DSc Degree

Publications 1982-1997

Michael D. Threadgill

Introduction

For the majority of my scientific career, I have been active in research using organic chemical methods and approaches to problems in biology and medicine, particularly as applied to the treatment and diagnosis of cancer. More than 70 papers have been published in refereed journals on this work, in addition to reviews and published contributions to conferences. One patent has been granted to me. As is usual for a medicinal chemist, the majority of my publications report both the chemical and biological aspects of the scientific work. For the publications with an essentially chemical theme, I led the research. For most of those with a more biological slant, I was the major chemical contributor to the collaboration; usually my medicinal chemical design led and shaped the direction of the project.

My work as a medicinal chemist has followed two principal themes, the chemistry and biology of *N*-methyl compounds and exploitation of the physiological differences between solid tumours and normal tissues. Several smaller projects have also been undertaken, often as parts of collaborations. A developing area of research is discovery of new potent and tumour-selective inhibitors of DNA repair.

This compilation of my published work comprises all my scientific papers in refereed journals from 1982 to 1997, together with my reviews, book chapters and a patent. Three published conference abstracts are also included where the scientific findings have not subsequently been reported elsewhere in full papers.

Chemistry, anti-cancer activity and metabolism of *N*-methyl compounds

My interest in the chemistry, anti-cancer activity and metabolism of *N*-methyl compounds was kindled in 1980 when the one of the simplest compounds to contain this group, *N*-methylformamide (NMF), was shown to have significant activity against murine tumours. This is a very small molecule (9 atoms only) and exploration of structure-activity relationships (SARs) was limited by the small number of functional groups that could be changed. Nevertheless, my team synthesised some 34 analogues but the structural requirements for antitumour activity were found to be very rigorous, only the parent being potent [*publication 15*]. This unusual SAR, together with the lack of myelosuppression caused by NMF, indicated that NMF was a very unusual antitumour agent in terms of its (bio)molecular mechanisms of therapeutic and toxic action. Studies were therefore initiated to establish the course and products of metabolism of NMF in experimental animals and to attempt to relate the chemical reactivity of the major metabolites to the therapeutic activity and toxicity.

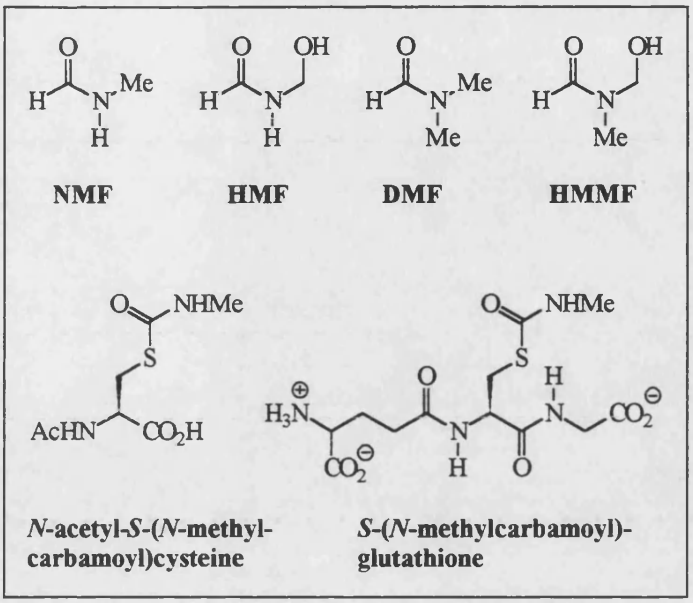
The established view at this time (early 1980s) was that most drugs and other agents containing *N*-methyl groups were metabolised by cytochrome P450-mediated oxidation at NCH_3, giving the *N*-hydroxymethyl analogue as a relatively unstable

intermediate and that the final observed metabolites were the *N*-desmethyl derivatives of the parents. Indeed, when *N*-[^{14}C]-NMF was synthesised [*publication 6*] and administered to mice in an early study, *N*-[^{14}C]-hydroxymethylformamide (HMF) was observed as a minor excreted metabolite [*publication 2*].

In parallel with these studies on NMF, discoveries were made concerning the metabolism of other *N*-methyl compounds, extending our understanding of their biological fate. *N,N*-Dimethylbenzamides, *N'*-aryl-*N,N*-dimethylformamidines, *N'*-aryl-*N,N*-dimethyltriazenes and *N'*-aryl-*N,N*-dimethylureas (including the agrochemical monuron) were synthesised; all were converted to their *N*-hydroxymethyl analogues by murine hepatic microsomes [*publication 1*]. However, a new discovery was made that where the *N*-hydroxymethyl metabolite was sufficiently stable, it could be oxidised further to the *N*-formyl metabolite [*publication 3,5*]. In contrast, metabolic reduction of the ketone carbonyl group of the experimental antitumour agent 1-(4-acetylphenyl)-3,3-dimethyltriazene was observed [*publication 4*]; selective chemical reduction of this group was also achieved in the presence of the normally reductively sensitive triazene moiety [*publication 7*]. During an attempt to synthesise 1-aryl-3-formyltriazenes as standards for these metabolic studies, we developed an efficient method for conversion of the arenediazonium compounds (and hence arylamines) to the parent arene, a process that is also amenable to regioselective introduction of deuterium and tritium [*publication 16*]. The metabolism of antitumour triazenes was reviewed [*publication 27, 64*]. Later, a trideuterio isotopomer of the experimental antitumour drug hexamethylmelamine was synthesised [*publication 45*]; biomimetic oxidation at the *N*-methyl groups was shown by mass spectrometry to be subject to a large primary kinetic deuterium isotope effect [KDIE] [*publication 53*].

N-Hydroxymethyl groups have the potential to form iminium ions under acidic conditions *in vitro* and the electrophilic reactivity of these ions may be important in the toxicity of *N*-methyl-containing compounds [*publication 13*] and in detoxifying conjugation reactions with, for example, glutathione. This electrophilic reactivity was also exploited in the chemical synthesis of a series of NCH_2S-linked glutathione derivatives of amides and ureas [*publication 9*], performed by three undergraduate project students under my supervision. These observations led to more fundamental NMR studies on the conformations of *N*-hydroxymethylamides in solution, establishing that hydrogen bonding is not the major determinant of rotamer population about the amide bond [*publication 10*], and a NMR, crystallographic and theoretical study on *N*-methyl-2-nitroethenamines closely related to NMF [*publication 11*], showing that hydrogen-bonding is very important in their structure. Where an *N*-hydroxymethyl compound is not sufficiently stable to be used in biological experiments, the *N*-acetoxymethyl analogue may be used as a source of iminium ions [*publication 19*]. We discovered that oxidation of *N*-aroylglycines (hippuric acids) with lead(IV) acetate conveniently gives both *N*-acetoxymethylbenzamides and *N*-formylbenzamides; the mechanism of this oxidative decarboxylation was also established using appropriate deuterium labelling experiments [*publication 17*].

The most important discovery in the field of the metabolism and toxicity of *N*-alkylformamides was that NMF does not follow the usual metabolic fate of *N*-methylamides. HMF, when administered to mice, does not cause significant hepatotoxicity but hepatotoxicity is the dose-limiting toxicity of NMF in rodents and in humans.



[^{14}C]-NMF gives rise to [^{14}C]-labelled protein adducts *in vivo* [*publication 21*], indicating that NMF is metabolised to an electrophile, but this electrophile was clearly not HMF. The search was started for the new metabolic pathway for *N*-alkylformamides that led to the hepatotoxic intermediate. The antitumour metabolite was also unidentified at this stage, NMF being devoid of cytotoxic activity *in vitro*. The first demonstration of this novel route came when *N*-acetyl-*S*-(*N*-methylcarbamoyl)cysteine was identified by proton NMR as a major urinary metabolite of NMF in mice; this compound had previously eluded identification owing to its high lability under even mildly basic conditions [*publication 18*]. Thus the major route of metabolism of NMF involved oxidation at the formyl group, rather than at the methyl group. This *N*-acetylcysteine derivative was likely to be a downstream (Phase 3) metabolite of the corresponding glutathione conjugate, *S*-(*N*-methylcarbamoyl)glutathione. Although this glutathione conjugate resisted chemical synthesis for some time [*publication 33*], it was identified as a biliary metabolite of NMF by mass spectrometry [*publications 23, 29*]. To investigate the metabolic pathways more thoroughly, I designed a series of experiments in which KDIEs would reveal information the mechanisms of the metabolic steps. A primary KDIE of approx. 6 was observed for the conversion NMF $\rightarrow$ *S*-(*N*-methylcarbamoyl)-glutathione, after co-administration of $OHCNHCH_3$ and $ODCNHCD_3$ to the mice. A similar KDIE was evident for the conversion of $OHCNHCD_3$ and $ODCNHCH_3$ to *N*-acetyl-*S*-(*N*-trideuteriomethylcarbamoyl)cysteine and *N*-acetyl-*S*-(*N*-methylcarbamoyl)cysteine, respectively. This was expected but more striking was the observation of a similar KDIE for the metabolism of $OHCNHCD_3$ and $ODCNHCH_3$ to CD_3NH_2 and CH_3NH_2, respectively, showing that the methylamine previously observed as a metabolite of NMF had also arisen from oxidation at the formyl group, rather than hydrolysis of the amide bond [*publication 23*]. Thus the major initial route of metabolism of NMF was oxidation at the formyl group; parallel studies also showed that this was the case for *N*-ethylformamide [*publication 22*]. *N*-Ethylformamide is a potent hepatotoxin in mice but has no antitumour activity. Interestingly, the various isotopomers of NMF and DMF were resolvable during capillary gas chromatography, deuterium incorporation leading to changes in retention time that were consistent and were diagnostic of deuteration at the methyl groups or at the formyl group [*publication 56*]. Publication ***23*** has proved to be my most highly cited paper to date.

Dimethylformamide (DMF), a common industrial chemical, is closely related in structure to NMF. It is largely devoid of antitumour activity in experimental activity, despite claims in the literature that NMF was the major metabolite of DMF. However,

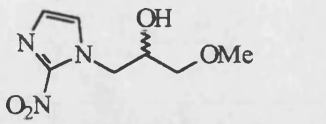
using proton NMR techniques directly on urine of mice which had received DMF, we were able to show that the major metabolite was, in fact, *N*-hydroxymethyl-*N*-methylformamide (HMMF) which decomposes to NMF under the gas chromatographic conditions previously used [*publication 14*]. DMF is an hepatotoxin in man but not in rodents and an explanation for this was sought in the metabolites downstream from HMMF. A sulphur-containing metabolite of DMF in the urine of environmentally exposed workers was shown to be *N*-acetyl-*S*-(*N*-methylcarbamoyl)cysteine, the same compound as was formed from NMF; thus the metabolic sequence DMF → HMMF → NMF → *S*-(*N*-methylcarbamoyl)glutathione → *N*-acetyl-*S*-(*N*-methylcarbamoyl)-cysteine was established in humans [*publications 22, 34*]. The step HMMF → NMF appeared to be much less efficient in rodents, correlating with the lower hepatotoxicity caused by DMF in these species. Clearly, either the primary metabolite of NMF (possibly methyl isocyanate) and / or *S*-(*N*-methylcarbamoyl)glutathione were acting as electrophilic toxins in the liver. A paper correlating the hepatotoxicity caused by certain low molecular weight formamides and acetamides with the extent of metabolic oxidation at the formyl group unified our work in this area [*publication 22*] and suggested that the health monitoring of occupationally exposed workers in the chemical industry at that time was inappropriate. Three chapters were also contributed to widely used reference texts in environmental toxicology and safety [*publications 30, 41, 42*]. The metabolic oxidation of DMF and of NMF at both the methyl and the formyl groups was shown to be mediated by one cytochrome P450 isozyme, CYP 2E1 [*publication 55*]; this is the isozyme which is responsible for the metabolism of ethanol and inhibition of ethanol metabolism by DMF and NMF may be responsible for the alcohol intolerance exhibited by some workers exposed to DMF.

The role of metabolic activation of formamides as hepatotoxins by oxidation of the formyl group to carbamoylating agents was confirmed by the profound isotope effect on hepatotoxicity when deuterium was present in the formyl group [*publication 23*]. In contrast, although the structure-antitumour activity relationships were very clear for the formamides, the identity of the therapeutically cytotoxic metabolite was never firmly established. Incorporation of deuterium at the formyl group did decrease the antitumour activity against some experimental tumours [*unpublished data*], suggesting that metabolic oxidation at formyl may also be required. Thus further drug design efforts to attempt to divorce the therapeutic activity from the toxicity may be futile. Unfortunately, no therapeutic activity could be demonstrated for NMF in phase I and phase II clinical trials; the formamide project thus came to an end.

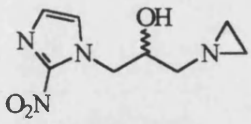
Medicinal chemistry of hypoxic solid tumours

Regions of chronic and acute hypoxia (oxygen deficiency) are present in most solid tumours owing to the primitive state of the tumour vasculature. Viable cells in such tissue are relatively resistant to radiotherapy and to many chemotherapeutic strategies. Thus hypoxia represents both a therapeutic problem (resistance) and a therapeutic opportunity, a physiological difference between normal and tumour tissue which could be exploited for selective delivery of drugs or activation of prodrugs. The medicinal chemical approach to overcoming the resistance of hypoxic cells to the lethal effects of X- and γ-radiation is to design an electron-affinic drug which will mimic the reactivity of molecular oxygen in abstracting electrons from ionised DNA. Much

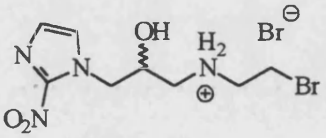
important work by Professor Gerald Adams and others in the 1970s and early 1980s had established that the radiosensitising potency of many types of drug correlated with their one-electron redox potential E^1_7. The Roche compound misonidazole had shown some radiosensitising activity in the clinic but doses were limited by neurotoxicity; incorporation of an electrophilic aziridine group into the side-chain increased the radiosensitising potency by about 10 fold in vitro but the new lead compound, RSU 1069, caused unacceptable gastrointestinal toxicity in the clinic. On joining the MRC Radiobiology Unit, my team continued the development of prodrugs of RSU 1069, contributing to the identification of the corresponding bromoethylammonium salt RB 6145 as the optimum prodrug which was equipotent with RSU 1069 as a radiosensitiser but which was tolerated by experimental animals at higher doses and permitted oral administration [*publication* ***39***].



Misonidazole



RSU 1069



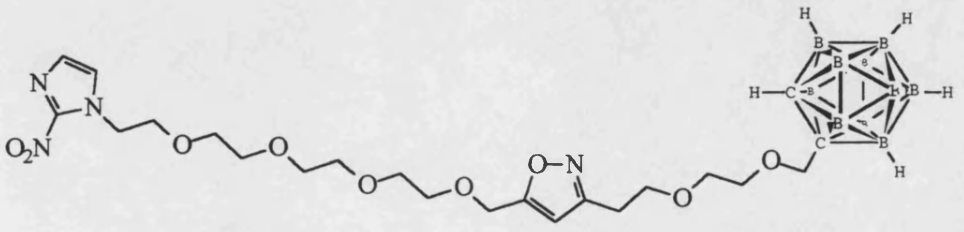
RB 6145

2-Nitroimidazoles, particularly those with electrophilic or weakly basic side-chain, were also selectively cytotoxic to hypoxic cells through metabolic reduction to electrophiles. A programme of synthesis and evaluation of other nitroheterocycles carrying electrophilic or tertiary amine side-chains largely confirmed the dependence of radiosensitising activity on redox potential but showed that selectivity and potency of the bioreductively activated cytotoxicity was largely confined to the nitroimidazoles; presumably the nitroheterocycles of higher redox potential were bioreduced in hypoxic cells but the reduction products were not cytotoxins [*publications* ***38***, ***46***, ***47***]. Interestingly, some of the *N*-(tertiary aminoalkyl)nitrothiophenecarboxamides showed radiosensitising activity considerably in excess of the efficiency predicted by their E^1_7 values.

Returning to the parent 1-substituted 2-nitroimidazole lead compounds RSU 1069 and RB 6145, a more detailed study of the effect on activity of small changes to the side-chains (introduction of methyl group at strategic points to vary the molecular conformations, pK_as, *etc.*) required some difficult synthetic obstacles to be overcome [*publication* ***51***]. Incorporation of the 3-aziridinyl-2-hydroxypropyl unit into a cyclohexane ring to lock the conformation was also investigated. During the course of the synthesis of the required 1,2,3-trisubstituted cyclohexane, a new method of controlling the diastereoselectivity of epoxidation reactions was developed in which the presence or absence of water would cause the reaction to proceed under steric or hydrogen-bonding control, respectively [*publication* ***44***]. This approach gave compounds which were equipotent with RSU 1069 or were marginally more potent; the increase in potency was more than outweighed in terms of drug development by the increased difficulty of synthesis. RSU 1069 and RB 6145 contain chiral centres and were developed as racemic mixtures. Surprisingly, synthesis and evaluation of the individual *R* and *S* enantiomers revealed no significant differences in therapeutic or toxic activity, in contrast to many drugs acting by binding to chiral targets such as DNA [*publication* ***57***].

2-[^{14}C]-2-Nitroimidazoles had previously been shown to be highly sensitive to auto-radiolysis, complete decomposition being seen in a few hours even at low specific activities. Hence, the requirement for biodistribution studies of tritiated isotopomers of RSU 1069, etanidazole and pimonidazole of high specific activity appeared to present a severe synthetic challenge - to carry out three steps of chemical synthesis and deliver the compounds for biological studies within 6 hours. Rapid and highly chemically and radiochemically efficient syntheses were developed to materials of 200 mCi mmol^{-1} [*publications **36**, **37**, **40***]. In contrast to the [^{14}C] isotopomers, these tritiated compounds proved to be stable for >1 year, permitting delivery to collaborating laboratories [*publication **54***].

The observation that the radioactivity associated with 1-substituted 2-nitroimidazoles is retained selectively in hypoxic tumour tissue led us to explore such compounds as tumour targetting systems. Boron neutron capture therapy of tumours relies on the capture of low energy "thermal" neutrons by the ^{10}B isotope of boron. The outcome of this capture process is fission of the boron nucleus, giving ^{7}Li and ^{4}He nuclei with sufficient kinetic energy to allow the α-particle to travel approximately one cell diameter in tissue. Thus, when tumour tissue is irradiated with thermal neutrons, damage is confined to those cells containing ^{10}B. Clinical studies of BNCT have met with mixed results, largely owing to lack of selectivity in the biodistribution of boron-containing drugs to tumours. In the early 1990s, we proposed that the selective retention of nitroimidazole derivatives may overcome this problem and a programme of synthesis and biological evaluation of nitroimidazoles linked to carboranes (clusters containing 10 boron atoms and 2 carbon atoms) was initiated. A number of difficulties were encountered in the syntheses, particularly associated with the instability of the nitroimidazole (an oxidising agent) to the boranes (reducing agents) required for assembly of the carboranes. My group was the first overcome these problems and to report an efficient process for construction of a nitroimidazole-carborane [*publication **49***]. Subsequent improvements in the 1,3-dipolar cycloaddition chemistry, which joined the nitroimidazole and carborane moieties under very mild conditions, led to the synthesis of more hydrophilic constructs which were acceptable for biological evaluation [*publications **60**, **63***]. New carbamate chemistry was also developed to enable this critical link to be made [*publication **61***]. In collaboration with Dr. Pauline Wood of the MRC Radiobiology Unit, the biodistribution of boron in tumour-bearing mice was measured non-invasively by ^{11}B NMR spectroscopy in vivo. We were the first group in the UK to use this technique in which sequential measurements can be made of biodistribution of boron with time in individual mice; these studies revealed that boron is indeed retained in (hypoxic) solid tumours but is cleared rapidly from several normal tissues, with the exception of liver [*publication **74***]. Isotopic labelling



Nitroimidazole-carborane with optimal biodistribution to tumour

of the leading nitroimidazole-carborane was achieved by development of a method involving highly selective deprotonation of the carborane 2-position [*publication 62*].

More recently, my group have sought to exploit selective bioreduction of nitroheterocycles in hypoxic tumours by incorporating such units into prodrugs. In one paper, we report that *S*-2-amino-5-(2-aminoimidazol-1-yl)pentanoic acid is a competitive inhibitor of nitric oxide synthases (NOS) but that the corresponding 2-nitroimidazole is a weaker inhibitor, leading to the possibility of tumour-selective NOS inhibition [*publication 81*]. However, this strategy does require that the structural element which is the product of bioreduction is necessarily a part of the active drug. Much more useful would be a general prodrug system which comprised the required active drug with critical structural features masked by a nitroheterocyclylmethyl unit. (Bio)reduction of the nitroheterocycle then triggers release of active drug. The elegance of this approach is that it will be possible to modify the physical properties, *e.g.* water-solubility, by attachment of appropriate functional groups to the bioreductive trigger moiety (the nitroheterocycle). In this way, formulation and biodistribution can be optimised without compromising the optimum structure of the delivered drug. We have synthesised nitrofuranylmethyl "prodrugs" of known DNA-repair inhibitors and BNCT agents and have demonstrated rapid release under chemically simulated bioreductive conditions [*publication 82*].

The vasculature of solid tumours is often poorly developed, particularly with respect to the structure of the walls of the vessels. The leakiness of the vessels, together with the lack of lymphatic drainage, leads to selective accumulation of soluble macromolecular material in tumours [*publication 77*]. The optimum hydrodynamic radius for this so-called enhanced permeation and retention (EPR) effect corresponds to molecules of 10-50 KDa, depending on structure. Selective delivery of cytotoxins to tumours has been achieved by other groups by attachment to non-biodegradable polyacrylamide polymers, leading to interesting clinical results. Our approach to delivery of drugs and imaging agents is to use a biodegradable alternating co-polymer of poly(ethylene glycol) and peptide units [*publication 86*]. In the course of this work, powerful new chemistry was developed for controlled attachment of porphyrins to the lysine and glutamic acid side-chains in the peptide units, giving the potential for selective delivery of paramagnetic manganese complexes for enhancement of contrast in magnetic resonance imaging [*publication 67*].

Serine hydroxymethyltransferase

Serine hydroxymethyltransferase (SHMT) is one of three enzymes in the folate cycle and catalyses the loss of a formaldehyde equivalent from serine, forming glycine in what is formally a reverse-Aldol reaction. The other two, dihydrofolate reductase (DHFR) and thymidylate synthetase (TS), have attracted the attention of medicinal chemists for many years and potent inhibitors of these enzymes are anticancer drugs widely used in the clinic. Inhibitors of this cycle inhibit *de novo* biosynthesis of DNA bases. In a short project, we attempted to design, synthesise and evaluate mechanism-based suicide inhibitors of SHMT, having established that the activities of the enzyme were much higher in tumours than in normal tissue [*publication 24*]. The first step in the mechanism is formation of an imine with the co-factor pyridoxal phosphate; cleavage of the α-β bond then forms a stabilised carbanion. A series of α-substituted

serines was synthesised in which formation of this carbanion was designed to lead to formation of an electrophile in the active site, inactivating the enzyme. Under my supervision, the graduate student achieved some challenging chemistry [*publications 26, 28*] but all analogues were found to be either non-inhibitory or to be competitive reversible inhibitors; molecular modelling showed a good fit of the most potent inhibitor, α-ethenylserine, with the active site [*publication 31*].

Flavones

The identification, in the early 1980s, of the receptor-associated tyrosine kinases and of the natural product quercetin (3,3',4',5,7-pentahydroxyflavone) as an inhibitor led us to initiate a project to attempt to develop new flavones which may be more potent and selective for this novel anticancer target. More than 30 substituted flavones were synthesised, using a mixture of classical and new methods [*publication 50*]. An NMR study on the intermediate 1-(2-hydroxyaryl)-3-arylpropane-1,3-diones revealed some interesting tautomeric and conformational information [*publication 35*]. The flavones were evaluated for their activity against the EGF receptor tyrosine kinase activity and for their selective cytotoxicity towards 3T3 cells transformed with the Abelson murine leukaemia virus. However, the cytotoxicity did not correlate well with inhibition of the kinase, suggesting an alternative mechanism of action for the optimum compound 3'-amino-4'-methoxyflavone.

Publication of the synthetic chemistry and these biological data led to many expressions of interest in the work and then to two valuable collaborations. Firstly, We supplied several of the more polar synthetic flavones to Dr. Wojciech Król of the Department of Immunology and Microbiology at the Silesian School of Medicine, Zabrze, Poland. Dr. Król and his group had been studying the effects of natural products on the function of macrophages as part of a larger programme on traditional therapies for inflammatory diseases. Several of the synthetic flavones were shown to inhibit the oxidative burst in stimulated murine macrophages, the most potent being 3'-amino-4'-hydroxyflavone [*publication 66*]. The profile of activities of the various flavones against generation of oxidising species by the macrophages was mirrored by the inhibitory activities against nitric oxide synthesis in these cells, suggesting a link between these two biochemical events [*publications 69, 71, 76*]. Secondly, in collaboration with Dr. Steven Safe of the Veterinary College, Texas A&M University, several of the substituted flavones were shown to be highly potent inhibitors both of CYP 1A1 (the arylhydrocarbon hydroxylase) and of the induction of this enzyme by the environmental pollutant tetrachlorodibenzodioxin (TCDD) [*publications 65, 71*]. The site at which these flavones compete with TCDD is the cytosolic arylhydrocarbon receptor which translocates to the nucleus when ligands are bound. Recent studies from another group have shown that this receptor protein may also be involved in the responses of cells to oxygen-deprivation and it is interesting to speculate that these antagonists may inhibit the protective adaptive response of malignant cells in hypoxic solid tumours.

Trifluoromethylheterocycles

The trifluoromethyl group has applications in new pharmaceuticals and in agro-chemicals, owing to its lipophilicity, acid-strengthening / base-weakening properties and its relative inertness to metabolism. As part of a collaboration with Professor Shiv P. Singh of Kurukshetra University, India, we were able to contribute to his structural and mechanistic work on the synthesis of trifluoromethylpyrazoles [*publications* ***48***, ***84***]. In my laboratory, we synthesised a series of trifluoromethyl pyrazoles, benzimidazoles and imidazopyridines and examined the effect of pH on their ^{19}F NMR spectra [*publication* ***75***]. Most of these showed a difference of 1-2 ppm in chemical shift between the neutral heterocycle and the conjugate base. For two examples, the pK_a for this equilibrium was near 7.0, *i.e.* near physiological pH. These compounds may therefore have applications in measuring pH in biological systems *in vitro* and *in vivo* by ^{19}F NMR, particularly if attached to suitable tissue- or cell-specific targetting systems such as nitroimidazoles. During the synthetic work, a novel cyclisation of 1,2,4-tris(benzamido)butene with trifluoroacetic anhydride to a 5-trifluoromethyloxazole was discovered [*publication* ***68***] and a serious error in the literature was identified and corrected [*publication* ***58***].

Poly(ADP-ribose)polymerase

Poly(ADP-ribose)polymerase (PARP) is abundant in all cell nuclei; it is an important regulatory enzyme and has a role in apoptosis. It catalyses the transfer of ADP-ribosyl units from NAD^+ to growing poly(ADP-ribose) chains attached to carboxylate residues in target proteins, including PARP itself. One of its major cellular functions is to control the repair of damaged DNA and inhibition of PARP leads to inhibition of repair of DNA damaged by chemotherapeutic drugs or by radiation [*publications* ***59***, ***78***]. PARP inhibitors can therefore act as chemopotentiating and radiopotentiating drugs. In a recently initiated programme, we have set out to design inhibitors which are more potent and more selective for tumour tissue [*publications* ***79***, ***80***].

Other chemical and biological research

During the course of my main themes of research a number of discoveries were made in synthetic and mechanistic chemistry. Chemical approaches were also used to contribute to a number of smaller collaborative projects.

An erroneous literature report on the reduction of 2-chloronitrobenzene with diethanolamine was corrected by careful separation and characterisation of the many products [*publication* ***12***]. 5-Aryl-2,4-diaminopyrimidines are potent inhibitors of DHFR and protonation is important to their mode of binding to the target and to their bio-inactivation and excretion; a ^{13}C NMR study revealed which nitrogen atoms were protonated and, in some cases, identified the order of protonation with decreasing pH of the medium [*publication* ***25***]. The azolotetrazinones, an important class of anti-tumour drugs including temozolomide (which shows good clinical activity against melanoma and gliomas) were discovered and developed at Aston University during the time that I was a member of the Cancer Research Campaign Chemotherapy Group

there. A comprehensive review of their chemistry and molecular mechanism of action was published in 1990 [*publication 43*].

In an attempt to exploit the different nutritional status of solid tumours, as compared to normal tissue, a mono(dichloroacetate) ester of glycerol was identified as a weak inhibitor of glycerol kinase [*publication 8*]. Naphtho-1,4-quinones, including vitamin K_1 and menadione, are hepatotoxins which had been proposed to act either by redox cycling or by acting as electrophiles. A series of 2,3-substituted naphtho-1,4-quinones was designed and synthesised and, in collaboration with Dr. David Ross, Dr. Peter Moldéus and Professor Sten Orrenius of the Karolinska Institutet, Stockholm, the contribution of each mechanism of toxicity was assessed [*publications 20, 32*].

A new and more efficient chemical derivatisation and HLPC analysis of cocaine and its metabolites was developed as a contribution to a research programme, led by Dr. Terry Jefferies of the University of Bath, on identifying and measuring drugs of abuse in human samples [*publication 52*]. Continuing the analytical theme, the synthesis of novel fluorescently tagged theophylline derivatives enabled a new automated immunoassay for theophylline to be set up for monitoring this widely used drug in biofluids [*publication 85*].

Methodological threads

Two methodological threads, NMR spectroscopy and isotopic labelling, run through the medicinal chemical programmes outlines above.

In addition to the traditional role of ^{1}H and ^{13}C NMR in characterisation of compounds synthesised, several papers report the use of NMR techniques, such as ^{1}H-^{1}H and ^{19}F-^{1}H nuclear Overhauser effect measurements in studying the stereochemistry, conformations and other structural properties of important molecules [*publications 10, 11, 25, 35, 44, 48, 68, 84*]. The biological applications of NMR have also been the principal or a subsidiary target in four project areas: direct ^{1}H NMR analysis of biofluids in for identification of drug metabolites [*publications 14, 18*], polymer-bound porphyrinatomanganese(III) for MRI [*publication 67*], development of a ^{19}F NMR method for determination of pH in biological samples [*publication 75*] and use of ^{11}B NMR *in vivo* for estimation of concentrations of boron in malignant and normal tissues [*publication 75*].

Isotopic labelling has been used for several applications in the chemical and biochemical programmes. Syntheses of molecules containing ^{2}H [*publications 6, 11, 16, 17, 23, 36, 37, 62*], ^{3}H [*publications 36, 37, 62*], ^{14}C [*publication 6*], ^{15}N [*publication 73*] and ^{18}O [*publications 72, 83*] have been achieved. Mechanisms of chemical reactions have been investigated by deuterium labelling at critical sites on the substrates or by following incorporation of deuterium from reagents; KDIEs have also given valuable chemical mechanistic information. Isotopomers incorporating radioactive nuclei (^{3}H and ^{14}C) were used in pharmacokinetics and drug metabolism studies. Most interesting was the use of specific deuterium labelling and KDIEs in elucidating metabolic routes of drugs.

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Michael D. Threadgill

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PUBLICATION 1

The Formation and Metabolism of *N*-Hydroxymethyl Compounds I - The Oxidative Demethylation of *N,N*-Dimethyl Derivatives of Arylamines, Aryltriazenes, Arylformamidines and Arylureas Including the Herbicide Monuron

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THE FORMATION AND METABOLISM OF *N*-HYDROXYMETHYL COMPOUNDS—I

THE OXIDATIVE *N*-DEMETHYLATION OF *N*-DIMETHYL DERIVATIVES OF ARYLAMINES, ARYLTRIAZENES, ARYLFORMAMIDINES AND ARYLUREAS INCLUDING THE HERBICIDE MONURON

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Abstract—The metabolism of the *N*-methyl moieties of aryldimethylamines and *N*-methyl compounds of the general formula Aryl-*X*-N(Me)$_2$, where *X* is either —N=N— (3-aryl-1,1-dimethyltriazenes), —NHCO— (*N'*-aryl-*N*,*N*-dimethylureas) or —N=CH— (*N'*-aryl-*N*,*N*-dimethylformamidines) was studied using mouse liver microsomes. Products of microsomal metabolism were reincubated with mouse liver homogenate devoid of microsomes and assayed colourimetrically for formaldehyde. This allows metabolically generated formaldehyde to be distinguished from formaldehyde precursors. Whereas the *N*-methyl moieties of the aryldimethyltriazenes, formamidines and amines were metabolised to formaldehyde, the aryldimethylureas formed stable formaldehyde precursors upon metabolism. The products of metabolism of one such aryldimethylurea, the herbicide monuron (*N'*-(4-chlorophenyl)-*N*,*N*-dimethylurea) were investigated using a high pressure liquid chromatographic method. Two metabolites were found on incubation of monuron with microsomes, one of which was identified as the *N*-desmethyl compound by mass spectrometry. The other product showed chromatographic properties similar to 4-chlorophenylurea but resembled the monomethylaryl urea on mass spectral analysis. It is concluded that this metabolite is likely to be *N'*-(4-chlorophenyl)-*N*-hydroxymethyl-*N*-methylurea. A urinary product of conjugative metabolism obtained after the administration of monuron to mice also gave the mass spectrum of the monomethyl compound after deconjugation which suggests that a conjugated *N*-hydroxymethyl compound may have been formed *in vivo*.

The oxidative *N*-demethylation of drugs which contain *N*-methyl groups (A in Fig. 1) is a metabolic pathway which is ubiquitous in xenobiochemistry. The pathway is considered to be initiated by the hydroxylation of the methyl carbon to form an N-hydroxymethyl or carbinolamine compound (Fig. 1, B). These *N*-hydroxymethyl compounds are generally thought to be unstable and to decompose to yield the *N*-desmethyl compound (Fig. 1, C) and formaldehyde [1]. It is probable that for the majority of *N*-methyl containing xenobiotics this reaction sequence occurs in the liver, an organ which is able to detoxify the formaldehyde, a species which might otherwise be toxic to peripheral cells [2].

Some xenobiotics do, however, undergo oxidative *N*-demethylation to yield relatively stable *N*-hydroxymethyl compounds, which are sometimes identified as their conjugates [3–11]. This is not altogether surprising given that the reaction between certain amines or amides and formaldehyde can give rise to characterisable, synthetic *N*-hydroxymethyl compounds of varying stability [12]. However, only certain amines and amides undergo this reaction, a fact which may be pertinent to the observation that relatively few *N*-hydroxymethyl compounds have been isolated after the metabolism of their progenitor *N*-methyl compounds.

We wished to determine those molecular features of *N*-methyl containing xenobiotics which might predispose them to form characterisable *N*-hydroxymethyl compounds and to estimate the stability of the latter. This was considered to be important for two reasons. Firstly, if stability was such that either the inherently reactive *N*-hydroxymethyl compound or the formaldehyde formed on its breakdown were available extrahepatically, such compounds may present a potential toxicological hazard to the host. Secondly, certain *N*-methyl containing antitumour drugs have been shown to form relatively stable *N*-hydroxymethyl compounds which have been implicated in their cytotoxicity [13]. Thus, an understanding of the molecular features which give rise to such compounds may be useful in predicting the structures of novel agents to be screened as potential antineoplastic drugs.

In this paper we report on the results obtained from a colourimetric assay [14] which distinguishes between free formaldehyde and its precursors such as *N*-hydroxymethyl compounds, formed from the *in vitro* metabolism of certain model compounds

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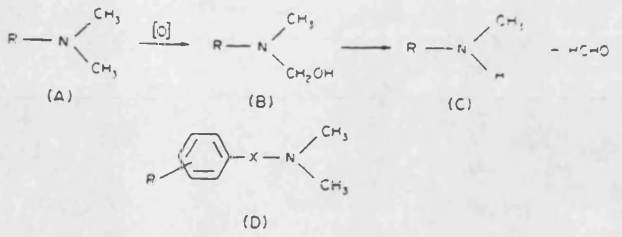


Fig. 1. Metabolism of *N,N*-dimethyl compounds.

which contain the *N*-methyl group. In addition, selected compounds have been subjected to HPLC (high pressure liquid chromatographic) analysis after their metabolism *in vitro*, in order to examine in more detail whether stable *N*-hydroxymethyl compounds had been formed. The model compounds where *X* equals —N=N— (triazenes), *X* equals —NHCO— (ureas), *X* equals —N=CH— (formamidines), or where *X* was absent (amines). Our rationale for choosing these particular types of compound was based primarily on previous studies of cytotoxic dimethylaryltriazenes where synthetic carbinolamines were shown to be relatively stable compounds [15], one of which was isolated as a conjugate in the urine of rats given a dimethylaryltriazene [11]. The ureas and formamidines are structurally similar to the aryltriazenes but the nitrogen bearing the methyl groupings is placed in different electronic and steric environments. The arylamines were chosen as simpler models and were considered appropriate to this study as evidence had been presented which suggests that a substituted arylamine, 3′-methyl-4-(methylamino)-azobenzene (MAB) may be metabolised to a carbinolamine [16].

MATERIALS AND METHODS

Animals and compounds. Male BALBc mice (20–25 g) were used for all metabolism experiments. The aryl dimethylamines (Ia–e Table 1) used in this study were obtained commercially. The 3-aryl-1,1-dimethyltriazenes (IIa–e) were prepared by treatment of the appropriate aryldiazonium salt with aqueous dimethylamine according to published procedures [17]. Condensation of anilines with dimethylformamide dimethylacetal, generally by published methods [18], furnished the formamidines (IIIa–f). IIIa, b, d, e were isolated as their tosylate salts.

Ureas (IVa, b, e, f, g) in addition to *N′*-(4-chlorophenyl)-*N*-methylurea (VI) and 4-chlorophenylurea (VII) were prepared by addition of a solution of the corresponding aryl isocyanate in diethyl ether or tetrahydrofuran to a large excess of ethereal dimethylamine. Melting points were consistent with published values [19–22].

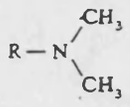
N′-(4-Cyanophenyl)-*N,N*-dimethylurea (IVc): 4-cyanobenzoic acid (5.88 g) was treated with boiling thionyl chloride (40 ml) and DMF (100 μl) for 2 hr. Evaporation of excess reagent gave the crude benzoyl chloride which, in acetone (50 ml), was added to 20% w/v aqueous sodium azide (80 ml). The mixture was stirred for 10 min, then extracted with ether (2 × 100 ml). The combined extracts were dried (Na_2SO_4), filtered and the solvents evaporated to give the crude benzoyl azide (ν_{max} (Nujol): 2220, 2180, 1690 cm^{-1}). This material, in dry toluene (100 ml), was stirred at reflux for 1 hr. The resulting solution of the isocyanate, from this Curtius reaction, was added to a tenfold excess of ethereal dimethylamine (300 ml). After 17 hr at ambient temperature, the solid was isolated and recrystallised from aqueous methanol to give the previously unreported *N′*-(4-cyanophenyl)-*N,N*-dimethylurea (4.90 g; 65%) as pale buff needles, MPt 152–153°. (Found: C 63.61%, H 5.63%; N 22.9%. Calculated for $C_{10}H_{11}N_3O$: C 63.48%; H 5.86%; N 22.21%.) ν_{max} (Nujol): 3410, 2260, 1730, 1600 cm^{-1}. $\delta((CD_3)_2SO)$: 7.75 (4H) s aryl-H, 7.1 (1H) br NH, 3.40 (6H) s $N(CH_3)_2$. *N*-(4-Trifluoromethylphenyl)-*N,N*-dimethylurea [23] was prepared similarly (from 4-trifluoromethylbenzoyl chloride) in 53% yield.

Assay for formaldehyde and its precursors. The metabolism of the *N*-methyl compounds was investigated essentially as described in [14]. In this assay substrates are incubated with hepatic microsomes for 30 min. At the end of the incubation period microsomes are precipitated by centrifugation and an aliquot of the supernatant is incubated with liver homogenate freed from microsomes. Metabolically generated formaldehyde is oxidised by the formaldehyde dehydrogenases which are abundant in mitochondria and liver cell cytosol, but virtually absent in microsomes [24]. Formaldehyde precursors, however, are not metabolised by these enzymes, and are detectable analytically as formaldehyde at the end of the incubation. Both formaldehyde and formaldehyde precursors were quantified by the colourimetric method according to Nash [25].

Substrate concentrations in the microsomal incubations varied between 0.5 and 5 mM according to the degree of *N*-demethylation of the substrates. Concentrations were used which gave substantial absorbance readings (>0.3 absorbance units) for Nash positive species at the end of the microsomal incubations. Control incubations were carried out without cofactors, and without substrate in the microsomal incubations. Substrates were also incubated with microsome free homogenate and NAD in the presence and absence of formaldehyde, to ensure that the substrates did not inhibit the removal of formaldehyde by aldehyde dehydrogenases, and

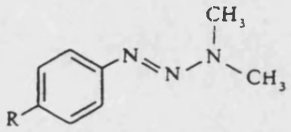
Table 1. *N,N*-Dimethyl compounds used in this study

Aryldimethylamines I



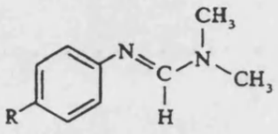
Ia: R = phenyl
b: R = 4-methylphenyl
c: R = cyanophenyl
d: R = pyridin-2-yl
e: R = pyridin-4-yl

Aryldimethyltriazenes II



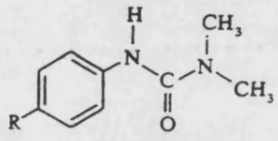
IIa: R = H
b: R = CF_3
c: R = Cl
d: R = $COCH_3$
e: R = CO_2CH_3

Aryldimethylformamidines III



IIIa: R = H
b: R = CH_3
c: R = CN
d: R = CF_3
e: R = Cl
f: R = SO_2NH_2

Aryldimethylureas IV



IVa: R = H
b: R = CH_3
c: R = CN
d: R = CF_3
e: R = Cl
f: R = Br
g: R = OCH_3

that the substrates themselves were not demethylated to Nash positive species by the microsome-free homogenate.

Incubation mixtures were deproteinized either by the addition of 20% trichloroacetic acid as described in [14], or in the case of incubations with acid labile triazenes as substrates with 0.6 ml of a 20% zinc sulphate solution followed by 0.6 ml of a saturated barium hydroxide solution. It is noteworthy to point out difficulties in the application of the colourimetric assay for formaldehyde to studies of the metabolism of the dimethyltriazenes. We found that the triazene derivatives IIa, d and e (Table 1) on reaction with Nash reagent formed species which absorbed at 412 nm, and thus superimposed the absorption of the chromophore produced from formaldehyde and Nash reagent. Interpretation was possible except in the case of IIe by using control incubations with triazenes and defining the conditions under which this led to high absorbance readings at 412 nm.

Metabolism of monuron. Metabolic incubations were performed in a final volume of 2.5 ml of Earls buffer (pH = 7.4) in which the final concentration of monuron was 1 mM. Cofactors were added to give a concentration of NADPH 1 mM and $MgCl_2$ 5 mM. Hepatocytes were prepared according to the method described in [26].

HPLC analysis of monuron and its metabolites. Metabolic incubates or urine samples were prepared for analysis by the addition of an equal volume of cold methanol containing internal standard (*N,N*-dimethyl-*N'*phenylurea), centrifuged and injected onto the HPLC column. Separation of the metabolites was performed on a 4.6 × 150 mm Ultrasphere ODS column (C_{18} reverse phase) using a linear gradient elution system from 10% methanol/water to 100% methanol over twenty minutes, a mobile phase flow rate of 1 ml/minute and a u.v. detection system (λ = 247 nm).

Chemical ionisation mass spectra. Chemical ionisation conditions were used as it was found that fewer contaminating peaks were seen in the spectra compared to those seen using electron impact. The mass spectra were determined on a VG 7070 mass spectrometer using isobutane as reagent gas. Spectra were run at a scan rate of 1 second/decade and were processed using a VG 2035 data system.

Mass numbers and percentage intensities of the major fragments in the mass spectra of compounds and metabolites referred to in the results section are as follows.

N'-(4-Chlorophenyl)-*N*-methylurea: m/z 185 (^{35}Cl-MH$^+$, 100%); m/z 187 (^{37}Cl-MH$^+$, 34.4%); m/z 168 (4.4%); m/z 151 (9.2%), m/z 127 (Cl-C_6H_4-NH_2^+, 13.2%). Metabolite with retention time identical with that of *N'*-(4-chlorophenyl)-*N*-methylurea on HPLC analysis : m/z 185 (100%); m/z 187 (36.2%); m/z 168 (6.6%); m/z 151 (8.7%); m/z 127 (14.2%). 4-Chlorophenylurea: m/z 171 (^{35}Cl-MH$^+$, 100%); m/z 173 (^{37}Cl-MH$^+$, 33.7%); m/z 127 (^{35}Cl-$C_6H_4NH_2^+$, 21.2%). Metabolite with retention time identical with that of 4-chlorophenylurea on HPLC analysis: m/z 185 (100%); m/z 187 (33.9%); m/z 168 (4.3%); m/z 151 (13.9%); m/z 127 (11.7%).

Deconjugation of urine samples. Urine samples were collected from mice in metabowl cages (Jencons, U.K.) after the injection of monuron 200 mg/kg i.p. as a suspension in 10% DMSO/arachis oil.

Enzymatic hydrolysis of urine samples was performed using 0.2 ml of urine diluted to 2 ml with acetate buffer (pH = 5) containing either β-glucuronidase (5000 u) or sulfatase (150 u sulfatase and

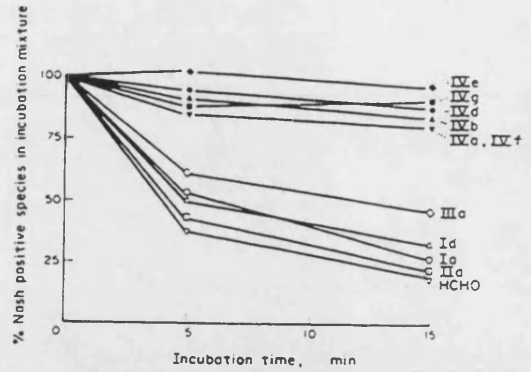


Fig. 2. Metabolism of Nash positive microsomal metabolites of dimethylaniline (Ia), 2-dimethylaminopyridine (Id), phenyldimethyltriazene (IIa), phenyldimethylformamidine (IIIa) and aryldimethylureas (IV a, b, d, e, g) by mouse liver homogenate free from microsomes. ∇ indicates disappearance of formaldehyde. Values are the mean of at least three experiments. Details of incubation conditions under Materials and Methods and in reference [14]. Results are expressed as percentage of the Nash positive species generated during the microsomal incubation after subtraction of control values obtained with incubations devoid of cofactors.

5000 u glucuronidase) (Sigma, U.K.). The samples were incubated at 37° for 17 hr and prepared for HPLC analysis as described above.

RESULTS

A number of *N*-methyl containing molecules (Table 1) were incubated with mouse liver microsomes and underwent oxidative *N*-demethylation to metabolites which gave a positive reaction with Nash reagent and were thus characterised as free formaldehyde or precursors of formaldehyde. Three compounds were not metabolized to species forming 3,5-diacetyl-2,6-dimethyl-1,4-dihydropyridine, the coloured chromophore in the Nash reaction: 4-dimethylaminopyridine (Ie), *N'*-(4-sulphonamidophenyl)-*N*,*N*-dimethylformamidine (IIIf) and *N'*-(4-cyanophenyl)-*N*,*N*-dimethylurea (IVc).

In order to test whether the species which gave a positive Nash reaction was free formaldehyde or a stable precursor of formaldehyde such as the *N*-hydroxymethyl metabolite B (Fig. 1), an aliquot of the microsomal incubate was reincubated with microsome-free liver homogenate as a source of formaldehyde oxidising enzymes. After 5 and 15 min the incubate was tested for residual Nash-positive species. Figure 2 shows the amount of such species obtained on incubation of compounds Ia, Id, IIa, IIIa, IVa–g, and formaldehyde, with microsome free liver homogenate. The Nash-positive metabolites of the 4-substituted derivatives of dimethylaniline Ib, Ic, phenyldimethyltriazenes IIb–d and phenyldimethylformamidine IIIb–e on exposure to the formaldehyde dehydrogenases in the microsome free liver homogenate behaved in essentially the same way as their unsubstituted congeners Ia, IIa, and IIIa and are not included in Fig. 2. The 4-substituted derivatives of phenyldimethylurea IVa–g formed metabolites which reacted with Nash reagent but were not substrates of formaldehyde metabolizing enzymes (Fig. 2). This result led us to conclude that the aryldimethylureas IV are metabolised to stable precursors of formaldehyde. In order to test if these precursors of formaldehyde were *N*-hydroxymethyl compounds, we subjected one derivative in this series, IVe, the herbicide monuron, to a more detailed metabolism study. Figure 3 shows the high pressure liquid chromatogram of a sample of the incubation mixture of monuron with mouse liver microsomes compared with a chromatogram of a solution containing reference compounds. Two metabolites were observed. On mass spectral investigation, one metabolite was identified as the *N*-demethylated derivative of IVe, *N'*-(4-chlorophenyl)-*N*-methylurea (VI, Fig. 4). The other metabolite in the chromatogram (Fig. 3) of the

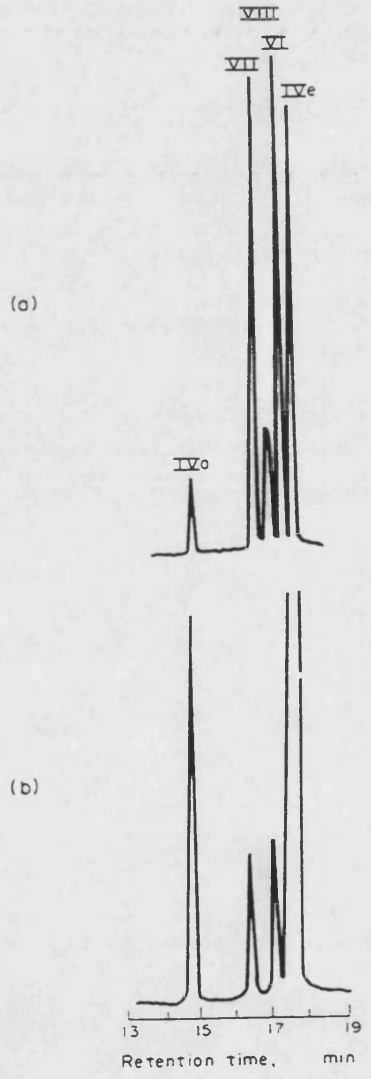


Fig. 3. High pressure liquid chromatogram of (a) a mixture of monuron (IVe), *N'*-(4-chlorophenyl)-*N*-methylurea (VI), 4-chloroaniline (VIII), 4-chlorophenylurea (VII) which were considered as possible metabolites and internal standard *N'*-phenyl-*N*,*N*-dimethylurea (IVa). (b) An extract of an incubation mixture of monuron (IVe) with mouse liver microsomes fortified with an NADPH generating system.

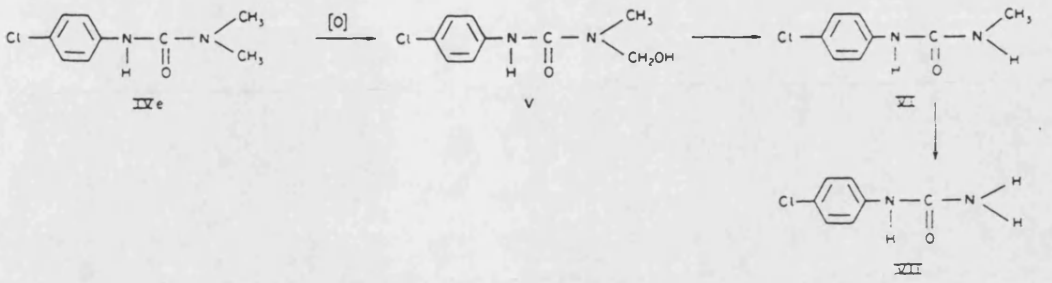


Fig. 4. Metabolism of monuron.

microsomal incubate had a retention volume similar to that of 4-chlorophenylurea (VII, Fig. 4). However, on treatment with acid or on heating the sample this metabolite decomposed with a corresponding increase in the amount of *N'*-(4-chlorophenyl)-*N*-methylurea VI. The chemical ionization mass spectrum of this unstable metabolite was identical with a synthetic sample of *N'*-(4-chlorophenyl)-*N*-methylurea. This suggested to us the possibility that the hydroxymethyl compound V (Fig. 4) had indeed been formed. This metabolite was also found on incubation of monuron with whole liver homogenate, 9000 g supernatant and isolated mouse hepatocytes. The major urinary metabolite of monuron was 4-chlorophenylurea VII, as identified by mass spectral investigation of the eluent obtained on high pressure liquid chromatography of a urine sample. After incubation of urine samples with a mixture of β-glucuronidase/sulfatase a metabolite was identified which on HPLC analysis (Fig. 5) and mass spectral comparison was identical with *N'*-(4-chlorophenyl)-*N*-methylurea VI.

DISCUSSION

There is no doubt that some xenobiotics containing *N*-methyl moieties are metabolised to identifiable *N*-hydroxymethyl compounds. Such species have been identified as metabolites of a number of *N*-methyl-amides [6, 7, 9], *N*-methyltriazenes [10, 11] and *N*-methylcarbazole [4]. The antineoplastic agents hexamethylmelamine and pentamethylmelamine were metabolized to formaldehyde precursors [14] and *N*-hydroxymethylpentamethylmelamine has been identified as the major *in vitro* metabolite of hexamethylmelamine [3]. Mueller and Miller [16] presented indirect evidence for the presence of an *N*-hydroxymethyl metabolite of the arylamine derivative 3'-methyl-4-(methylamino)-azobenzene (MAB). Their interpretation was based on the metabolic production of formaldehyde and on the ability of glutathione to react with a metabolic intermediate to yield a water soluble azo dye which could be hydrolyzed subsequently to the water insoluble *N*-demethylated aminoazo dye. It may be relevant that in all of these cases the *N*-methyl group is attached to molecules of electronegative character.

We investigated the *in vitro* metabolism of arylamine derivatives Ia–e (Table 1), one of which has the strongly electron-withdrawing cyano group in the 4-position of the aryl moiety (Ic) and which is

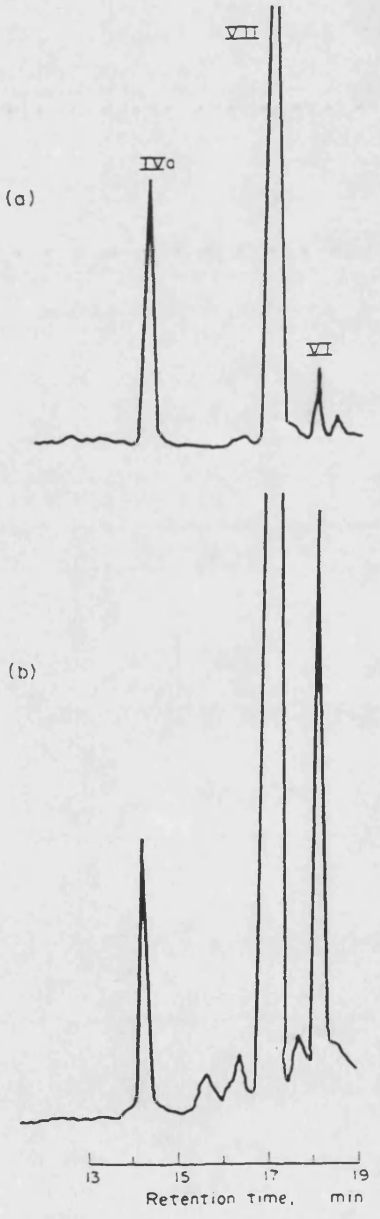


Fig. 5. High pressure liquid chromatogram of (a) an extract of urine of mouse after administration of 200 mg/kg monuron i.p., (b) an extract of a sample of the same urine after incubation with glucuronidase/sulfatase. For the explanation of the identifying numbers see legend to Fig. 3.

mutagen like MAB [27], but could not find evidence for the metabolic generation of formaldehyde precursors of measurable stability. The yellow colour of substituted aminoazobenzene derivatives such as MAB interfered with the colourimetric assay and prevented investigation of the metabolism of these compounds. Changing the arylamine structure I (Table 1) by introducing a —N=N— or a —N=CH— moiety into the molecule between the *N,N*-dimethyl and the aryl portions of I, leads to the triazenes II and the formamidines III which were metabolised to species which, like the metabolites of the *N,N*-dimethylarylamines I, behaved biochemically like formaldehyde (Fig. 2). The failure of the assay to detect stable *N*-hydroxymethyl metabolites of dimethyltriazenes is puzzling, in view of the reported isolation of a glucuronide of an *N*-hydroxymethyl-*N*-methyltriazene as a urinary metabolite of an aryl dimethyltriazene in the rat [11]. This may be due to species differences in metabolism, or may reflect a lack of conjugation in this microsomal system which could stabilise any *N*-hydroxymethyl metabolites when produced. Whatever the explanation the difficulties involved in predicting the metabolic behaviour of compounds *in vivo* from results obtained using *in vitro* model systems must be stressed.

The only compounds tested in this study which formed stable formaldehyde precursors were the urea derivatives IV (Table 1). These molecules differ from the *N,N*-dimethylarylamines I in that they contain an —NH—CO— structure inserted between the aryl ring and the *N,N*-dimethylamino group which reduces the electron density at the nitrogen atom bearing the *N*-methyl group. Evidence that the stable formaldehyde precursors were *N*-hydroxymethyl compounds was corroborated by the results of the chromatographic and mass spectral investigation of an *in vitro* metabolite of *N'*-(4-chlorophenyl)-*N,N*-dimethylurea (IVe, monuron). This metabolite behaved chromatographically like 4-chlorophenylurea (VII, Fig. 4) but its mass spectrum identified it as *N'*-(4-chlorophenyl)-*N*-methylurea (VI, Fig. 4). We suggest, therefore, that it is *N'*-(4-chlorophenyl)-*N*-hydroxymethyl-*N*-methylurea (V, Fig. 4). The glucoside conjugate of this compound (V) was reported as a product of the metabolism of monuron in cotton plants [28], but our results are the first indication that this *N*-hydroxymethyl metabolite of monuron is generated in animals. That a urinary metabolite of monuron on enzymatic deconjugation was identified as the monomethyl compound VI (Fig. 5) is also compatible with the suggestion that its precursor was the carbinolamine V. It is conceivable that V after hydrolysis of its glucuronide or sulphate derivatives decomposes on incubation at 37° in acetate buffer (pH = 5) to the monomethyl derivative VI. Alternatively this precursor may be a conjugate of VI linked to the conjugating species via either of the two nitrogen atoms.

All derivatives of IVa, except IVc, irrespective of the electron-withdrawing (IVd, e, f) or electron-donating (IVb, g) nature of the substitute formed metabolites which were formaldehyde precursors but not substrates of formaldehyde metabolising enzymes (Fig. 2). It therefore appears that the nature of the substitute in the aryl ring of IV does not determine whether metabolism leads to a stable carbinolamine or directly to formaldehyde, likewise the variation of substituents in the 4-position of the aryl moieties of the aryldimethyltriazenes II and the aryldimethylformamidines III does not change the instability of their metabolic *N*-hydroxymethyl intermediates. It is likely that several physicochemical factors influence the equilibrium which the carbinolamine B (Fig. 1) maintains with formaldehyde [29] and its is possible that of all the factors involved the electronic environment of the *N*-hydroxymethyl moiety as influenced by para substitute in the aryl ring is only of minor importance.

We have recently investigated the stability of the *N*-hydroxymethyl derivative of formamide, $OHC\text{-}NHCH_2OH$, and have found that it is surprisingly stable and does not react as a formaldehyde precursor unless treated with strong alkali [30]. Similarly, *N*-hydroxymethylbenzamide, ($C_6H_5\text{-}CO\text{-}NHCH_2OH$) has been reported to be very stable at physiological pH [31], and we have evidence that *N*-hydroxymethylbenzamide is indeed a metabolite of *N*-methylbenzamide ($C_6H_5\text{-}CO\text{-}NH\text{-}CH_3$). These recent findings (to be presented in a future report) and the results discussed above underline the fact that the oxidative metabolism of the *N*-methyl moiety in xenobiotic molecules is more complex than simply a pathway leading to the *N*-desmethyl compound and formaldehyde. Depending on as yet unknown factors associated with the structure of the molecule metabolites with *N*-hydroxymethyl groups can be generated with widely different stabilities under physiological conditions. Those of intermediate stability may decompose to liberate formaldehyde at extrahepatic sites or may participate in endogenous metabolic pathways as 'active formaldehyde' [32], or may aminomethylate biologically important targets. A recent report of the metabolism of MAB suggests that further reactions of a conjugated methylol may be relevant to the carcinogenicity of MAB [33] and we would suggest that the putative carbinolamines formed from other *N*-methyl containing carcinogens, such as dimethylnitrosamine, may similarly contribute to their toxicology, a hypothesis actively under investigation by us. Finally, it is also pertinent to stress the inadequacy of the colourimetric determination of formaldehyde when used to assess the metabolic *N*-demethylation of those substrates forming very stable *N*-hydroxymethyl compounds which do not react in the Nash test for formaldehyde.

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PUBLICATION 2

Studies of the Pharmacology of *N*-Methylformamide in Mice

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Cancer Treatment Reports, **1982**, *66*, 1957-1963.

Studies of the Pharmacology of *N*-Methylformamide in Mice[1,2]

Charles Brindley, Andreas Gescher,* Ernest S. Harpur, David Ross, John A. Slack, Michael D. Threadgill, and Helen Whitby[3,4]

When 400 mg/kg of ^{14}C-methyl-labeled *N*-methylformamide (NMF) was injected ip into mice, the curve for plasma concentration of radioactivity versus time was superimposable on the curve obtained by measuring unmetabolized NMF with gas-liquid chromatography during the first 24 hrs. Radioactivity in plasma was measurable for 8 days after NMF administration, but NMF was not measurable by gas chromatography beyond 24 hrs after administration. Radioactivity was eliminated from the plasma after 60 hrs, with an apparent half-life of 71.1 hrs. Of the radioactivity injected with NMF, 73.6% was recovered in the urine in 24 hrs; 26.4% of this was unchanged NMF. Three percent of the administered radioactivity was exhaled as $^{14}CO_2$ in 7 hrs at a constant rate of 0.007% per min. One urinary metabolite was a stable precursor of formaldehyde, which decomposed to formaldehyde only after alkaline hydrolysis and may well be *N*-(hydroxymethyl)-formamide. The areas under the plasma concentration versus time curve were estimated after ip, iv, and oral administration of NMF. The bioavailability of NMF was 1.01 after oral administration and 1.10 after ip administration. [Cancer Treat Rep 66:1957–1965, 1982]

The antitumor activity of *N*-methylformamide (NMF) ($OHC\text{-}NHCH_3$) against murine tumor models was first described by Clarke et al in 1953 (1). In a clinical trial, NMF was found to cause reversible liver damage in all seven patients in whom it was tested (2). We have recently found NMF to be active against the mouse ovarian reticulum cell sarcoma M5076 and the mouse TLX5 lymphoma (3). The animal tumors that are sensitive to NMF do not respond to treatment with other *N*-alkyl derivatives of formamide or with formamide itself.

The mechanism by which NMF exerts antitumor activity is unknown. We found NMF to be cytotoxic only at concentrations > 0.5 M against TLX5 lymphoma cells in an in vitro-in vivo bioassay (3). In this bioassay, *N*-ethylformamide (the ethyl analog of NMF), which is devoid of antitumor activity in vivo, exhibited cytotoxicity comparable to that of NMF.

Since NMF possesses antitumor activity against a considerable number of murine tumors and does not appear to be toxic to the bone marrow (4), it is a candidate for further clinical evaluation. To gain information that may facilitate the rational use of NMF in clinical trials, several aspects of its disposition in the mouse were studied. ^{14}C-Methyl-labeled NMF (^{14}C-NMF) was synthesized and the plasma disposition of radioactivity was compared with that of unlabeled NMF.

Renal excretion and metabolism to CO_2 are two obvious routes of elimination that a small molecular weight compound like NMF can undergo. Therefore, the appearance of radioactivity after administration of ^{14}C-NMF was studied in the urine and the breath. Special attention was given to identifying products of the oxidative metabolism of NMF in the urine, in view of our finding that formamide is a urinary metabolite (3).

The bioavailability of NMF after ip or oral administration was also investigated because in the antitumor tests, NMF was administered ip, whereas in the clinic, NMF may be given orally as well as iv or im. Finally, the influence of the presence of the TLX5 lymphoma on the plasma disposition of NMF was investigated.

MATERIALS AND METHODS

NMF and formamide were purchased from Aldrich Chemicals (UK); *N*-(hydroxymethyl)-formamide was synthesized by Mr. E. N. Gate in our laboratories ac-

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[4]We thank Dr. J. A. Hickman for his interest in this work and Karen Ensor for her help with the chromatographic determination.
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cording to a published method (5). ^{14}C-NMF was prepared by treatment of ^{14}C-methylamine hydrochloride (Radiochemical Centre, Amersham, UK) with ethyl formate and sodium carbonate; details of the synthetic procedure will be published elsewhere. Drugs were administered in solution in sterile saline. Male CBA CA mice (20–25 g) were used throughout this study. Approximately 10^5 TLX5 cells from routine passage as ascites were inoculated sc in the inguinal region of 20-g mice.

Measurement of NMF and total radioactivity in the plasma and urine.—The drug was injected into a tail vein iv, administered ip, or given by esophageal intubation. The doses administered were 400 mg/kg (equivalent to 1/2 × LD10 determined by ip administration) and 80 mg/kg. The amount of radioactivity injected into each mouse with the dose was either 5 or 15 μCi. While mice were under halothane anesthesia, blood samples (20 μl) were collected into heparinized capillary tubes from the tip of the tail for at least ten time intervals following drug administration. In a control experiment, blood was obtained without anesthetizing the animals, and plasma concentration versus time profiles were not different from profiles derived from anesthetized animals. Plasma was obtained by centrifugation, and samples were diluted with acetone and centrifuged again. Urine was collected from mice kept in metabolic cages (Jencons, UK). To minimize the risk of evaporative losses, the urinary collection funnels were washed with 10 ml of water after 2–6-hr intervals during the first 12 hrs and again 24, 48, and 72 hrs after drug administration. For the analysis by gas chromatography (GC), 10 μl of blood or 100 μl of diluted urine was further diluted with 50 or 200 μl of acetone (including internal standard). Radioactivity was counted after adding 10 μl of blood or 1 ml of diluted urine to 5 or 10 ml of NE260 Micellar scintillant (New England Nuclear, W Germany), in a Packard Tricarb 2660 scintillation counter using the external standardization mode. The centrifuged plasma or urine samples containing acetone with tetramethylurea as internal standard were analyzed by GC as described previously (3). The limit of detection for NMF was 3–5 μg/ml.

Breath analysis.—After iv administration of ^{14}C-NMF, the mice were placed in air-tight metabolic cages and the exhaled radioactivity was collected continually in a trapping fluid that was renewed at frequent intervals as previously described in reference 6. The trapping fluid did not contain measurable amounts of unmetabolized NMF as indicated by GC analysis, so that most of the exhaled radioactivity was considered to be in the form of $^{14}CO_2$.

Pharmacokinetic analysis.—The decline in plasma concentration of radioactivity with time beyond 24 hrs after drug administration was multiexponential. The decline of plasma concentration of radioactivity beyond 60 hrs after administration of ^{14}C-NMF was considered to represent the elimination of drug or metabolites, and in each animal the plasma concentration values obtained beyond 60 hrs after ^{14}C-NMF administration were subjected to a linear regression analysis that afforded a line (correlation coefficient > 0.98) from which a half-life was computed. The areas under the plasma concentration versus time curves (AUC) for unchanged NMF up to 24 hrs after NMF administration were estimated by the trapezoidal rule. AUC and half-life values as well as recoveries of NMF and radioactivity in the urine, as given in the Results section, are the mean ± SD from at least five animals. The bioavailability was calculated, using mean AUC data, from the ratio $\frac{\text{AUC ip or oral}}{\text{AUC iv}}$.

Analysis of formaldehyde precursors in the urine.—Urine samples were diluted and made alkaline by the addition of 1 N NaOH (to pH 12), and liberated formaldehyde was measured colorimetrically according to Nash (7).

RESULTS

NMF (400 mg/kg) was injected ip and its concentration in plasma was measured by GC and compared with the plasma disposition of radioactivity after administration of ^{14}C-NMF. Figure 1 shows that the plasma concentration versus time plot of NMF as measured by GC is very similar to that of total radioactivity during the first 24 hrs after drug administration. This suggests that unchanged NMF is the main constituent of the total radioactivity measured in the plasma. GC estimation of NMF in plasma samples collected beyond 24 hrs after NMF administration was unreliable since levels were generally near or below the detection limit of the assay. Radioactive species derived from ^{14}C-NMF were, however, measurable in the plasma for 8 days after drug administration (fig 2). The apparent concave downward curvature of the line up to 24 hrs on a semilog plot (fig 1) suggests that the disappearance of the drug from plasma does not obey first-order kinetics during this time. However, the disposition of radioactivity beyond 24 hrs can be described by a curve that appears to exhibit first-order kinetics with at least two exponential components (fig 2). Radioactivity was detectable in the plasma up to 192 hrs after administration of ^{14}C-NMF. The decline of plasma concentration of radioactivity with time between 60 and 192 hrs after drug administration is linear and may well represent the elimination of radioactivity, ie, of NMF and/or its metabolites. (Elimination rate constant K_{el} = 0.0097 ± 0.0012 hr^{-1}; elimination half-life = 71.1 ± 8.8 hrs.)

A total of 73.6% ± 6.8% of the radioactivity was recovered in the urine within 24 hrs after ip administration of 400 mg/kg of ^{14}C-NMF. Figure 3 shows that

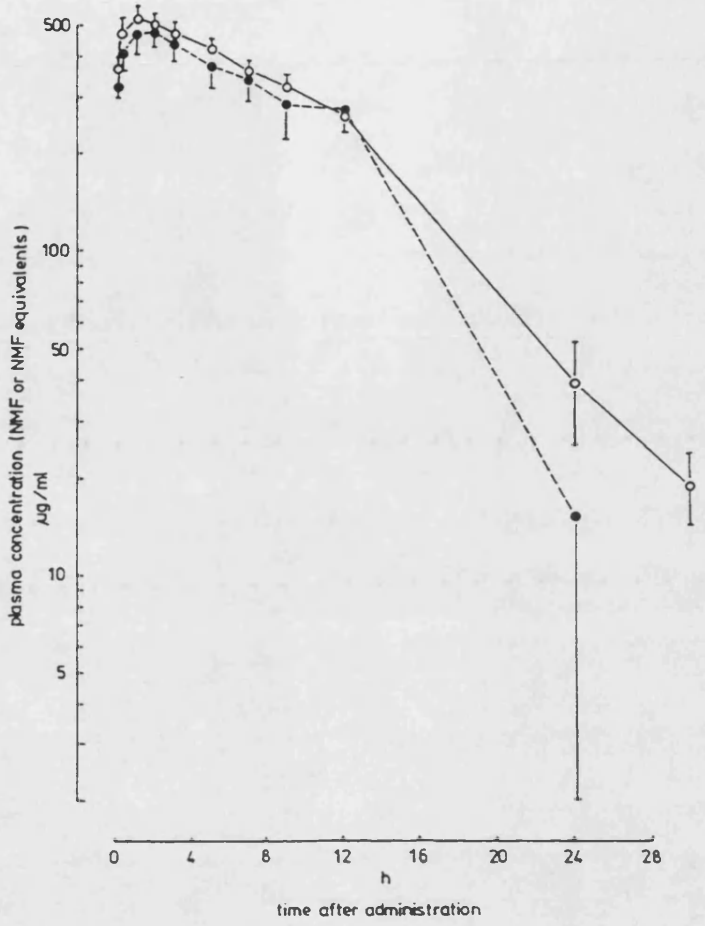


FIGURE 1.—Plasma disposition of NMF measured by GC (●) and of total radioactivity (○) after ip administration of 400 mg/kg of NMF or ^{14}C-NMF. Concentrations of radioactivity are expressed as NMF equivalents. Values are the mean ± SD of 6 experiments, except for the point without an error bar, which is the mean of 3 experiments.

26.4% ± 2.9% of the radioactivity in the urine was unchanged NMF and < 2% (not shown in fig 3) of the dose was eliminated as either formamide, the *N*-demethylated metabolite of NMF, or its *N*-hydroxymethyl precursor, *N*-(hydroxymethyl)-formamide ($OHCNHCH_2OH$); the latter also appears as formamide on GC determination. Some urinary metabolites (or a metabolite) of NMF were hydrolyzed to formaldehyde when urine samples were treated with NaOH, and the hydrolysate reacted with Nash reagent. In two experiments, 4.4 µmols of the metabolites (expressed as formaldehyde equivalents) were excreted with the urine in 36 hrs. *N*-(Hydroxymethyl)-formamide releases formaldehyde on alkaline hydrolysis, so the urinary formaldehyde precursor may well be this carbinolamine. Control animals and those that received *N*-ethylformamide did not excrete formaldehyde precursors sensitive to alkali.

Radioactivity exhaled with the breath as $^{14}CO_2$ after administration of 400 mg/kg of ^{14}C-NMF was collected for 7 hrs. Only 3% of the radioactivity injected was exhaled during this time, at a constant rate of 0.007%/min.

When NMF was administered iv or orally, the disappearance of drug from plasma with time did not substantially differ from the disappearance of NMF from the plasma after ip injection. In figures 4 and 5, graphs of NMF plasma concentrations plotted on a linear scale against time after administration of two iv (fig 4) and oral (fig 5) doses of NMF (80 and 400 mg/kg) reveal apparently linear sections, which indicate that zero-order kinetics might partially govern these plasma disposition profiles. The time required for plasma levels to decline from peak concentrations to 50% after oral administration of NMF (fig 5) was 9.5 hrs for the high dose and 8 hrs for the low dose.

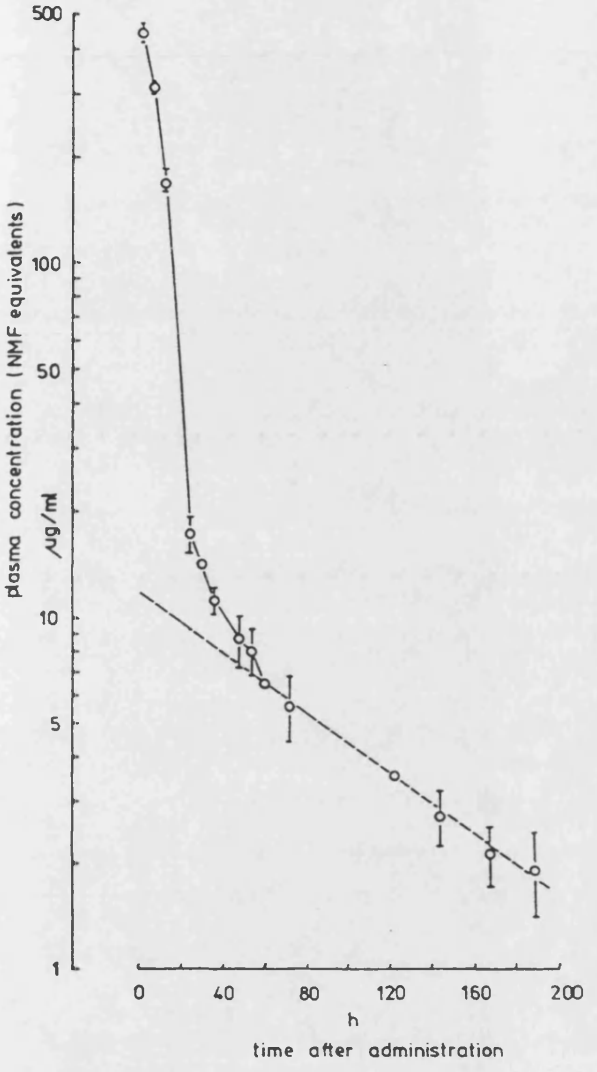


FIGURE 2.—Plasma disposition of radioactivity after ip administration of 400 mg/kg of ^{14}C-NMF. Values are the mean ± SD of 6 experiments; the values without error bars are the mean of 3 experiments. Dashed line indicates the line obtained by linear regression analysis of points beyond 60 hrs after drug administration.

AUC values were calculated up to 24 hrs after administration of 400 mg/kg of NMF and were 5235 ± 1270 $\mu g \cdot hr \cdot ml^{-1}$ after iv administration, 5842 ± 783 $\mu g \cdot hr \cdot ml^{-1}$ after ip administration, and 5360 ± 1117 $\mu g \cdot hr \cdot ml^{-1}$ after oral administration. Thus, the systemic availability of NMF was 1.01 after oral administration and 1.10 after ip injection.

Mice were inoculated with the TLX5 lymphoma, which is sensitive to NMF (3). Five days after tumor implantation (approximately half the lifespan of untreated tumor-bearing mice), a plasma concentration versus time profile was determined after ip injection of NMF in these mice. It was virtually identical with the profile seen in normal mice. The AUC calculated from the profile in six tumor-bearing mice was 6595 ± 564 $\mu g \cdot hr \cdot ml^{-1}$ and did not differ significantly from the value obtained in normal mice.

DISCUSSION

Radioactivity injected as a single dose of ^{14}C-NMF (400 mg/kg) was detected in plasma for 8 days after drug administration, and the plasma radioactivity concentration versus time curve (fig 2) shows that a drug disposition phase that can be described by first-order kinetics was not reached until 24 hrs after NMF administration. After 60 hrs, radioactivity was cleared from the plasma with an apparent half-life of 71.1 hrs.

The physicochemical nature of NMF and its potential metabolites has so far prevented the unequivocal identification of the radiolabeled species present in this late phase of NMF disposition; the high water:octanol partition coefficient of NMF and its likely metabolites (unpublished results) prevents efficient extraction into organic solvent and their high volatility renders freeze-drying of samples inappropriate.

Repeated daily administration of high doses of NMF was required for optimal antitumor activity in murine tumor models (3), and it is likely that to be effective in patients, NMF may have to be given in high doses on multidose schedules or by constant infusion. If the long elimination half-life of drug-derived species observed in mice (fig 2) is also found in man, then repeated dosing at frequent intervals will lead to drug accumulation. Whether accumulation of NMF or its metabolites is a prerequisite for its antitumor efficacy or the generation of its hepatotoxicity remains to be elucidated.

The plasma disposition profile of radioactivity during the first 24 hrs after drug administration is virtually superimposable on that of unlabeled NMF (fig 1), which shows that only a small amount of metabolite(s), if any, appears in the plasma during this time. However, metabolites were found in the urine (fig 3), which suggests that after their formation, the metabolites (whatever their chemical nature) are rapidly eliminated into the urine.

The plasma concentration versus time curves of NMF (as measured by GC) within 24 hrs after ip (fig 1), iv, and oral administration of 400 mg/kg exhibit a concave downward curvature when plotted semilogarithmically. This curvature was not seen in a preliminary study of NMF plasma levels (3) when blood was obtained by cardiac puncture. The mathematical treatment of these data, based on linear kinetics, might not be appropriate and the concept of plasma elimination rates and half-lives cannot be used to describe these curves. When plotted using Cartesian coordinates, concentration val-

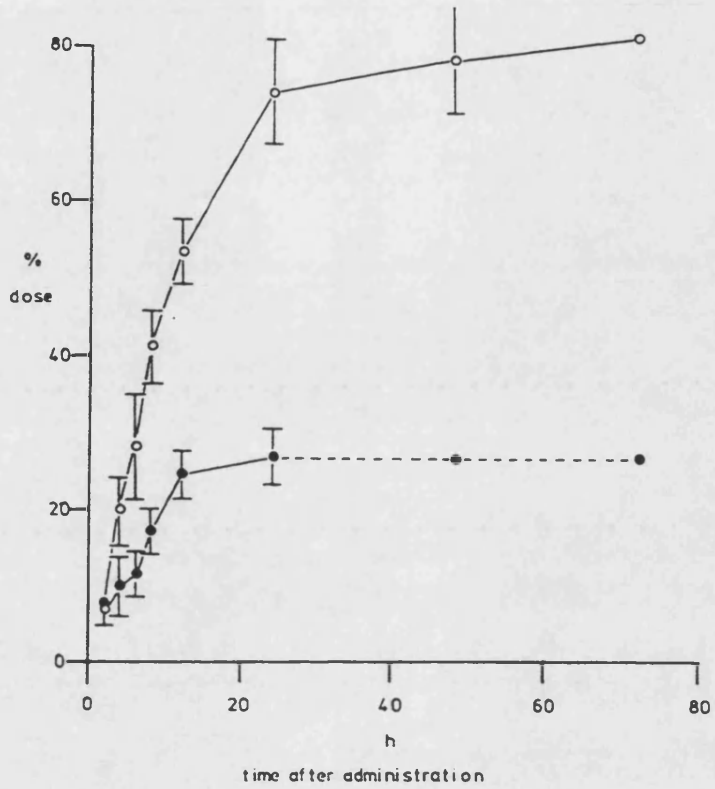


FIGURE 3.—Cumulative urinary excretion of NMF measured by GC (●) and of total radioactivity (○) after ip administration of 400 mg/kg of NMF or ^{14}C-NMF, expressed as % of dose administered. Values are the mean ± SD of 5 experiments. There were no measurable levels of unchanged NMF in the urine after 24 hrs.

ues during some periods of the disposition appear to fit a straight line (figs 4 and 5), which indicates that NMF might be eliminated at a fixed rate independent of its concentration in the plasma (zero-order kinetics). Nonlinear pharmacokinetics are often associated with the saturation of the pathways by which a drug is distributed in or eliminated from the body, eg, uptake into tissues or hepatic metabolism (8). The data in figs 4 and 5 suggest either that both doses are above the saturable point or that the removal process is zero-order regardless of the dose in the first 24 hrs. When plasma concentration values obtained within 24 hrs after administration of 80 and 400 mg/kg of NMF are divided by the dose and the ratios are plotted against time (fig 6), the resulting curves, although not superimposed, are not conspicuously different from each other [nonlinear kinetics must be suspected if the curves are not superimposable (8)]. This does not facilitate a conclusion as to whether or not the plasma disposition of NMF is governed by nonlinear kinetics. No further attempt has yet been made to determine which model best describes the plasma disposition of NMF. Further studies are required to unambiguously establish nonlinearities in the pharmacokinetics of NMF. To define the kinetic model for the disposition is important because it may allow the prediction of drug levels in the body and, as a consequence, the prediction of therapeutic or unwanted drug effects.

Most of the radioactivity injected with NMF was excreted into the urine within 24 hrs (fig 3). Only 26.4% of the dose appeared in the urine as unchanged NMF and < 2% as formamide [or *N*-(hydroxymethyl)-formamide]. To identify the urinary metabolite(s) of NMF is relevant in view of the suggestion that NMF may require metabolic activation to exert its antineoplastic activity (3). Since the metabolite(s) were cleared from

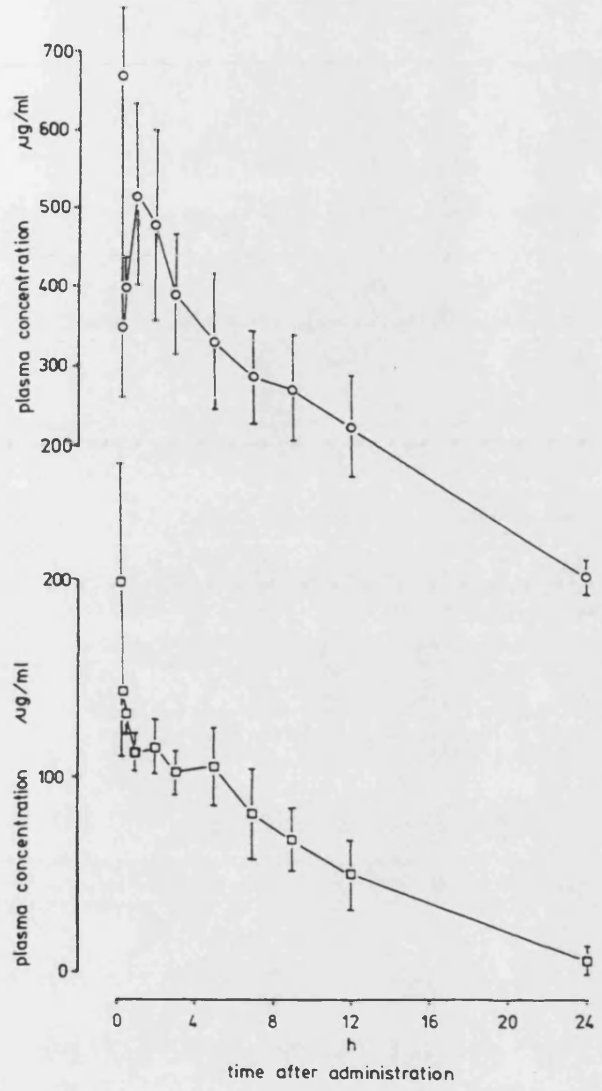
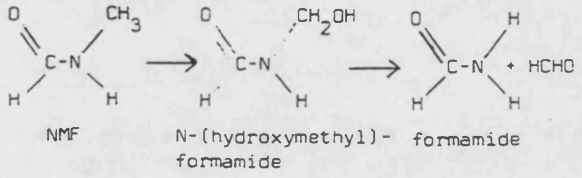


FIGURE 4.—Plasma disposition of NMF after iv administration of 80 mg/kg (□) or 400 mg/kg (○) of NMF. Values are the mean ± SD of at least 5 mice.

the blood rapidly, it is possible that they are very hydrophilic conjugates. Indeed, on alkaline hydrolysis, urine of NMF-treated mice contained a large amount of thiol compounds, suggesting the presence of metabolites with thioether structure, such as *N*-acetylcysteine derivatives (A. Gescher, unpublished observation). One metabolite was characterized as a stable precursor of formaldehyde, presumably *N*-(hydroxymethyl)-formamide. Preliminary unpublished results show that this species does not possess the antitumor efficacy of NMF and does therefore not appear to be an active metabolite. The evidence for the presence in urine of *N*-(hydroxymethyl)-formamide as a metabolite of NMF suggests the following metabolic scheme:



It is puzzling that radioactivity excreted with the breath as $^{14}CO_2$, the final oxidation product of the NMF methyl moiety, was exhaled at a constant rate of 0.007%/min during 7 hrs after drug administration. The $^{14}CO_2$ exhalation pattern observed with ^{14}C-NMF contrasts with the profiles reported after administration of other drugs that undergo metabolic *N*-demethylation, eg, hexamethylmelamine, aminopyrine, and caffeine. In these cases, the $^{14}CO_2$ exhalation profiles reflect the plasma disposition of drug-derived *N*-methyl moieties (6). It is possible that in the case of NMF, the *N*-hydroxymethyl metabolite is so stable that further metabolism (eg, cleavage of the N-C bond to form formaldehyde), since it does not occur spontaneously, is catalyzed by an enzyme that is saturated on administration of large doses of NMF. This might lead to a slow zero-order formation of CO_2, probably circumventing free formaldehyde in the metabolic process, at a rate that is independent of plasma NMF concentration and that in turn determines the rate at which $^{14}CO_2$ appears with the breath.

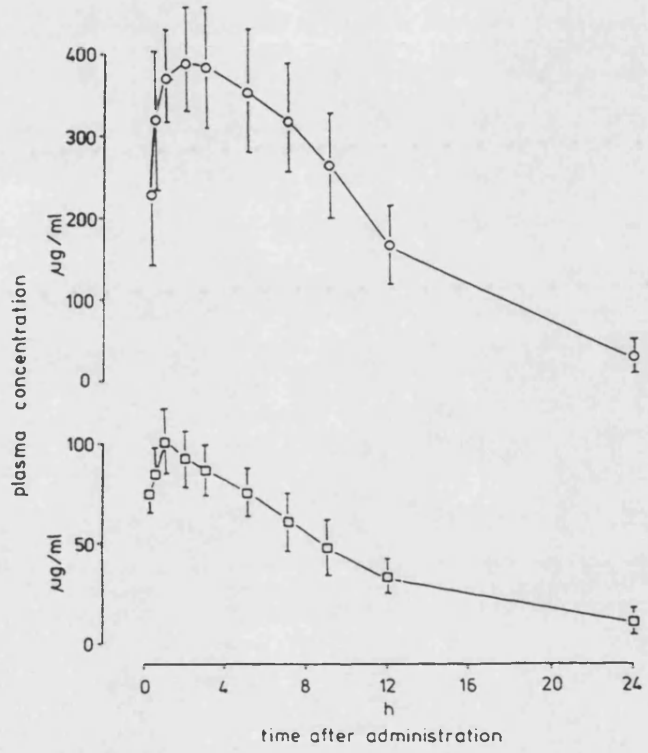


FIGURE 5.—Plasma disposition of NMF after oral administration of 80 mg kg (●) or 400 mg/kg (○) of NMF. Values are the mean ± SD of 4–8 mice.

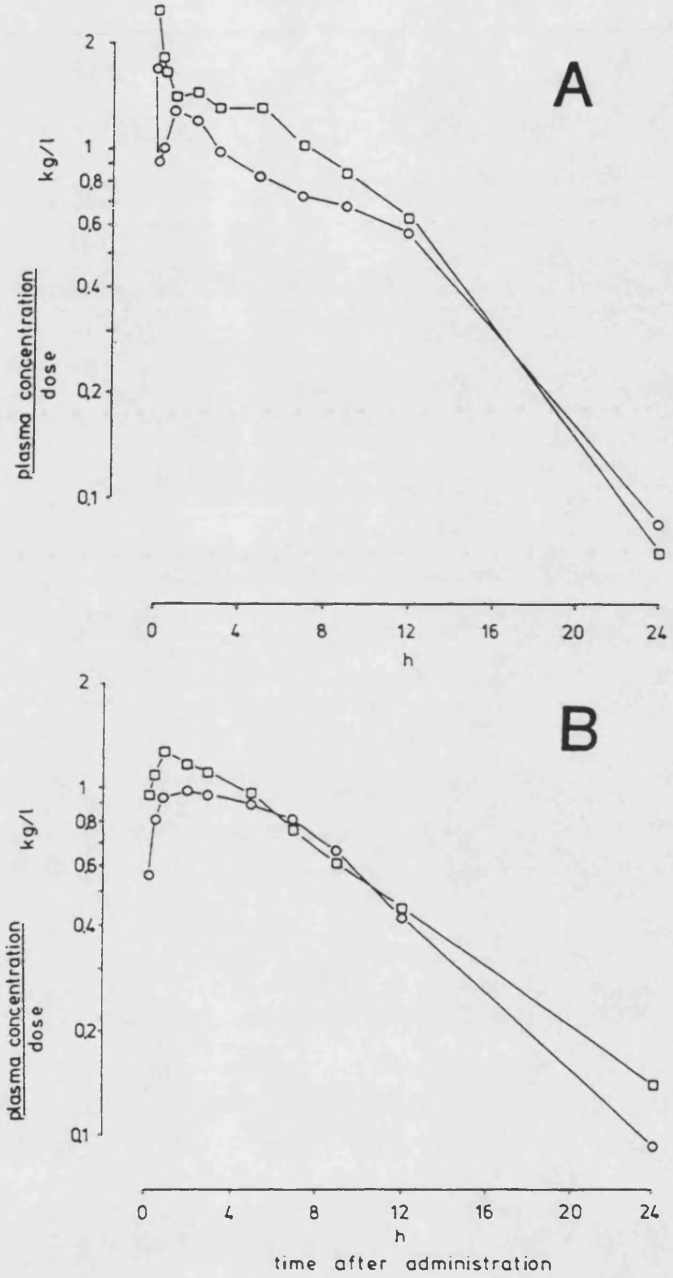


FIGURE 6.—Plots of NMF plasma concentrations divided by the dose against time after iv (A) or oral (B) administration of either 80 mg/kg (□) or 400 mg kg (○) of NMF. Values are identical with the mean values shown in figs 4 and 5.

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PUBLICATION 3

The Metabolism of a Stable *N*-Hydroxymethyl Derivative of a *N*-Methylamide

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THE METABOLISM OF A STABLE N-HYDROXYMETHYL DERIVATIVE OF A N-METHYLAMIDE[1,2,3]

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Summary

N-Formylbenzamide and benzamide were characterised by high pressure liquid chromatography and mass spectrometry as products of the metabolism of N-hydroxymethylbenzamide in incubation mixtures with mouse liver preparations and isolated hepatocytes. This biotransformation occurred predominantly in 9000g and microsomal supernatant fractions and was also catalyzed by horse liver alcohol dehydrogenase fortified with NAD and could be inhibited by pyrazole. Unlike N-hydroxymethylbenzamide, which is very stable, N-formylbenzamide degraded rapidly to benzamide in buffer at pH 7.4 with a half-life of 7.8 min. The instability of N-formylbenzamide and the time course of its metabolic generation together with benzamide suggest that benzamide is a chemical breakdown product of N-formylbenzamide. N-Formylbenzamide was also tentatively identified as a urinary metabolite of N-hydroxymethylbenzamide. This is the first time that an N-hydroxymethyl compound has been shown to undergo metabolism either *in vitro* or *in vivo*.

Certain N-methyl containing xenobiotics, particularly those with a N-methyl moiety situated in an electron-withdrawing environment, form stable N-hydroxymethyl compounds on metabolism *in vitro* (1,2). These stable carbinolamines may be of toxicological importance because they may be transport forms of the cytotoxic agent formaldehyde (3). In general, N-hydroxymethyl compounds decompose rapidly to formaldehyde and the N-des-(hydroxymethyl)amine or amide. The metabolic fate of stable N-hydroxymethyl compounds is however not known. We therefore investigated the metabolism of a representative of such an N-methylol, N-hydroxymethylbenzamide, which possesses a half life of 1.1×10^4 min in buffer at pH 7.4 and 37°C (4) and is a major *in vitro* metabolite of N-methylbenzamide (D. Ross *et al*, manuscript in preparation). We report here that N-hydroxymethylbenzamide undergoes further metabolism and is biotransformed to N-formylbenzamide, an unstable species which breaks down chemically to benzamide.

[1]The previous publication in this series is reference No. 2.
[2]Supported by a grant from the Medical Research Council. The authors thank John Lamb for the determination of mass spectra.
[3]Presented in part at the 8th European Workshop on Drug Metabolism, Liege, Belgium, September 1982.
[4]To whom reprint requests should be addressed.

Materials and Methods

Chemicals and animals. N-Hydroxymethylbenzamide was kindly provided by Dr H. Bundgaard, Royal Danish School of Pharmacy, Copenhagen, Denmark. N-Methylbenzamide, benzamide and pyrazole were purchased from Aldrich Chemical Co., Dorset, U.K. N-Formylbenzamide was synthesised in our laboratories essentially according to published methods (5). Glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (GPDH), nicotinamide adenine dinucleotide phosphate (NADP), and its reduced form, NADPH, and equine liver alcohol dehydrogenase were purchased from Sigma U.K., Poole. Male Balb C mice (20-25g) were used for all experiments.

In vitro metabolism studies. Livers were excised, washed and homogenised in 0.25M sucrose to give a 20% w/v homogenate. Liver fractions were prepared according to the method of Cinti et al (6). In all experiments the amount of each liver fraction used was equivalent to 0.25g of liver (wet weight). Incubations were performed in Earl's buffer (pH 7.4) and were fortified with either 1 mM NAD, 0.5 to 1 mM NADPH and 5 mM magnesium chloride or sufficient G6P, GPDH and NADP to yield 0.5 to 1 mM NADPH.
Hepatocytes were prepared according to the method of Renton et al (7) and incubations performed essentially as described in (8). Reactions were started by the addition of N-hydroxymethylbenzamide in 0.1ml acetone to give a concentration of 1 mM in a final incubation volume of 2.5ml. When purified horse liver alcohol dehydrogenase was used as the enzyme source, 1.8 units were used per incubation with or without the addition of pyrazole 1 mM Incubations were performed in a final volume of 2.5ml Earl's buffer (pH 8.8) for thirty minutes at 37°C, with a three minute pre-incubation period prior to the addition of substrate. At the end of the incubation period the reactions were stopped by immersing the incubate in ice, 0.15ml of p-nitrobenzamide (200µg/ml in acetone) was added as an internal standard, and the incubate was then extracted twice with 4ml of ethyl acetate. The extracts were evaporated to dryness under a stream of nitrogen at room temperature, re-dissolved in 300µl of 30% methanol/water and subjected to HPLC analysis.

In vivo metabolism studies. 0.1ml N-Hydroxymethylbenzamide in 10% DMSO/ arachis oil was injected intraperitoneally into mice at a dose of 200mg/kg. Control mice received only the vehicle. Urine was collected using metabolic cages (Jencons, U.K.) over a period of 24h. 0.04ml of p-nitrobenzamide solution (500µg/ml) was then added to 0.5ml of both control and sample urine. The urine was then extracted twice with 1.5ml ethyl acetate and prepared for HPLC analysis as described above.

HPLC analysis. Separation of metabolites was performed using an Altex 100A pump and a Waters radial compression unit equipped with a C_{18}-5µ reverse phase column. The mobile phase was 30% methanol/water, flow rate 2ml min^{-1}, and a U.V. detection system was used (λ=254nm). Column eluates were collected, extracted with ethyl acetate, concentrated under a stream of nitrogen, re-dissolved in acetone and subjected to mass spectral analysis.

Chemical ionisation mass spectra. Chemical ionisation conditions were used because a common solvent contaminant (m/z 149) interfered with the molecular ion produced from N-formylbenzamide when spectra were determined in the electron-impact mode. The mass spectra were determined on a VG7070 mass spectrometer using isobutane as reagent gas. Spectra were run at a scan rate of 1 second/decade and were processed using a VG2035 data system. Samples were admitted to the mass spectrometer (source temperature 200°C) via the direct insertion probe.

Recoveries of standard compounds. Recoveries of N-hydroxymethylbenzamide, N-formylbenzamide, benzamide, and p-nitrobenzamide from incubation mixtures and urine were greater than 87%.

Results

An HPLC method which separates N-hydroxymethylbenzamide and its derivatives was developed and used to investigate metabolites formed on incubation of N-hydroxymethylbenzamide with isolated hepatocytes and with various mouse liver fractions. Benzamide and N-formylbenzamide were the two major metabolites and Fig. 1 shows the extract of an incubation mixture containing 9000g supernatant fortified with NAD. The chromatographic eluate corresponding to the peak with the retention time of authentic N-formylbenzamide was collected, and the mass spectra of this metabolite and N-formylbenzamide were very similar, thus confirming that this metabolite was N-formylbenzamide. The three major fragments in the mass spectra of the reference compound and the metabolite together with their intensities (%) and assignments are: N-formylbenzamide, m/z 105 (100%, $C_6H_5CO^+$), m/z 150 (76.8%, MH^+), m/z 122 (19.1%, $C_6H_5C(OH)NH_2^+$); metabolite, m/z 105 (100%), m/z 150 (56%), m/z 122 (10.9%).

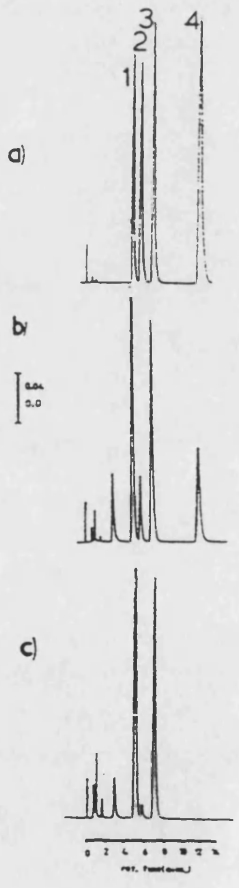

FIG. 1

High pressure liquid chromatogram of a) a mixture of the following reference compounds 1. N-hydroxymethylbenzamide, 2. benzamide, 3. p-nitrobenzamide (internal standard), 4. N-formylbenzamide, b) an extract of a mixture of N-hydroxymethylbenzamide with liver 9000g supernatant and NAD, incubated for 30 min. c) an extract of an incubation mixture omitting NAD.

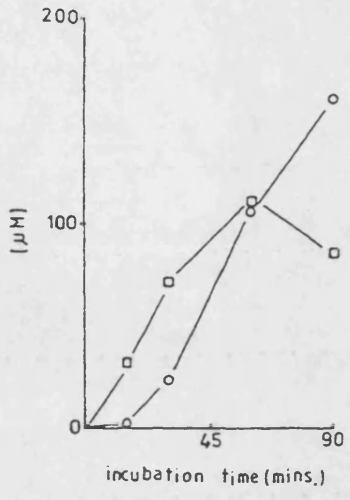

FIG. 2

Time course of the metabolic generation of N-formylbenzamide (squares) and benzamide (circles) from N-hydroxymethylbenzamide (1mM) in incubations with mouse hepatocytes. The mixtures (2.5ml) contained $5x10^5$ cells/ml. The values were obtained in a typical experiment and very similar graphs were obtained with hepatic 9000g supernatant.

TABLE I

Metabolism of N-Hydroxymethylbenzamide (1mM) in Various Liver Fractions and Hepatocytes

Liver Fraction (equivalent to 250 mg of liver)	Amount of Metabolites Formed (N-Formylbenzamide and Benzamide)(μM)
Mitochondria	22 ± 29
9000g Supernatant	249 ± 53
Microsomes	25 ± 21
Microsomal Supernatant	152 ± 55
Hepatocytes*	273 ± 31

Liver fractions, but not hepatocytes, were fortified with 1mM NAD and incubated at 37°C for 30 min. *$5x10^5$ hepatocytes/ml were incubated for 90 min. The values are the mean ± S.D. of at least three experiments.

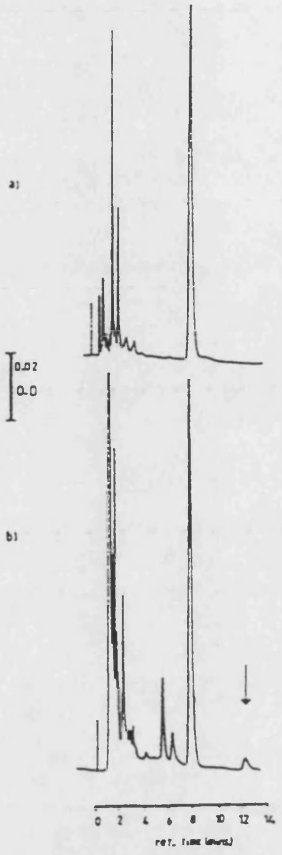

FIG. 3

High pressure liquid chromatogram of a) an extract of control urine and b) an extract of urine from mice which had received N-hydroxymethyl-benzamide (200 mg/kg). The arrow indicates a peak with a retention volume identical to that of authentic N-formyl-benzamide.

TABLE II

The Effect of Pyrazole (1mM) and Nitrogen on the Metabolism of N-Hydroxymethyl-benzamide(1mM) by 9000g Supernatant and Horse Liver Alcohol Dehydrogenase (HLAD).

Enzyme Source	Amount of Metabolites Formed (N-Formylbenzamide and Benzamide)(μM)
9000g Supernatant	211 ± 11
9000g Supernatant + Pyrazole	0
9000g Supernatant + Nitrogen	97 ± 38
HLAD	309 ± 22
HLAD + Pyrazole	0
HLAD + Nitrogen	364 ± 15

Mixtures were incubated including NAD 1mM at 37°C for 30 min. Values are mean ± S.D. of at least three experiments.

When authentic N-formylbenzamide was incubated at 37°C in Earl's buffer, pH 7.4, it hydrolysed quantitatively to yield benzamide (and presumably formic acid) with a half-life of 7.8 ± 1.9 min. This half-life was not altered in the presence of 9000g supernatant and NAD indicating that in this system the production of benzamide from N-formylbenzamide is purely a chemical process. This suggests that any benzamide formed during metabolism may be a product of the degradation of N-formylbenzamide and the time-course of production of these two metabolites using isolated mouse hepatocytes (Fig. 2) supports this hypothesis.

The biotransformation of N-hydroxymethylbenzamide to N-formylbenzamide and benzamide was observed in isolated hepatocytes, 9000g supernatant, and microsomal supernatant but only to a minor extent in microsomal and mitochondrial fractions (Table I). The rate of metabolism in the liver supernatant preparations was greatly enhanced by the addition of either NAD, NADPH, or an NADPH generating system. The addition of pyrazole, an inhibitor of alcohol dehydrogenase (9), to the 9000g supernatant completely abolished the metabolic oxidation of N-hydroxymethylbenzamide (Table II) which suggests the involvement of dehydrogenase enzymes in this process. Purified horse liver alcohol dehydrogenase fortified with NAD catalysed the biotransformation of N-hydroxymethylbenzamide which was also abolished in the presence of pyrazole. When incubations were performed in an atmosphere of nitrogen however, the products of metabolism were decreased by 50% in fortified 9000g supernatant but remained unchanged in the case of the purified enzyme. When benzamide (1 mM) was used as a substrate no N-formylbenzamide could be detected in any of the metabolising systems used.

Fig. 3 shows a chromatogram of an extract of urine from mice which had received N-hydroxymethylbenzamide. The presence of a peak which corresponds to the retention volume of authentic N-formylbenzamide and its absence in the chromatogram of the control urine suggests that the formation of N-formylbenzamide may also occur *in vivo*. Confirmation of the identity of this minor peak in the chromatogram by mass spectrometry was not attempted.

Discussion

The conversion of N-alkylamines to carbinolamines and further to amides is a known metabolic transformation and has been demonstrated e.g. for cyclophosphamide (10) where the carbinolamine is a pharmacologically active and the amide an inactive species (11). In the case of N-methyl drugs metabolically generated carbinolamines are often fugitive agents and decompose to formaldehyde. Some N-methyl-containing xenobiotics have been shown to undergo metabolism to N-formyl compounds e.g. aminopyrine (12, 13) and chlorotoluron (14), and in the latter case the N-formyl metabolite was suggested to be an intermediate in the demethylation process.

N-Hydroxymethylbenzamide is a metabolite of N-methylbenzamide and has been unequivocally shown here to be metabolised to N-formylbenzamide. Thus we have demonstrated for the first time that an N-hydroxymethyl metabolite of an N-methylamide can be further metabolised to an N-formyl compound. Such a mechanism has been suggested as a possible route of activation for the carcinogens dimethylnitrosamine and methylazoxymethanol (15) and may therefore be of toxicological importance.

The observation that N-formylbenzamide is not detected when benzamide is used as a substrate in metabolic incubations, suggests that any N-formylbenzamide produced arises from the biotransformation of the N-hydroxymethyl group and not as a result of metabolic N-formylation. In view of this finding and the fact that both N-hydroxymethylbenzamide and N-formylbenzamide can be

detected as metabolites of N-methylbenzamide using isolated mouse hepatocytes (D. Ross, unpublished observation), we suggest the metabolic scheme in Fig. 4 for the N-methyl group in N-methylbenzamide. Although N-hydroxymethylbenzamide does not break down chemically under these conditions, enzymic production of benzamide cannot be ruled out. N-Hydroxymethyl compounds are able to aminomethylate nucleophiles (16) and this process has recently been implicated as the mechanism whereby an N-methylene-glutathione conjugate was formed after administration of the carcinogen dimethylaminoazobenzene to rats (17).

The enzymes which catalysed the conversion of N-hydroxymethylbenzamide to N-formylbenzamide and benzamide were localised predominately in the soluble fraction of the cell. The results suggest that dehydrogenases may be the principal enzymes involved, although the non-specific co-factor requirements and the effect of nitrogen suggest that more than one enzyme system may be responsible.

Finally, it is pertinent to note that the N-formyl metabolite produced in this case is less stable than its N-hydroxymethyl precursor and decomposes to yield the corresponding amide. This shows that in certain cases both the N-hydroxymethyl and the N-formyl species can be intermediates in metabolic N-demethylations, and that N-demethylation itself need not be synchronous with formaldehyde production.

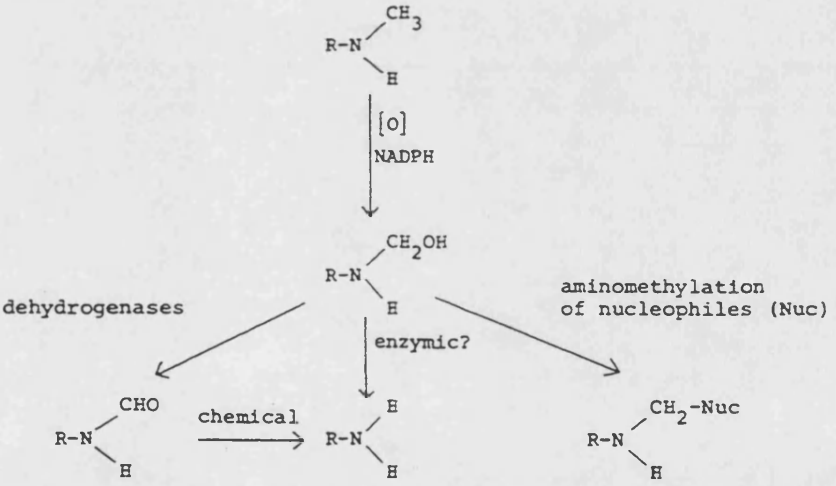

FIG. 4

Metabolic scheme for N-methylbenzamide (R = benzoyl)

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PUBLICATION 4

Metabolism of the Anticancer Agent 1-(4-Acetylphenyl)-3,3-dimethyltriazene

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Metabolism of the Anticancer Agent 1-(4-Acetylphenyl)-3,3-dimethyltriazene

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High pressure liquid chromatography was used in combination with mass spectrometry to confirm that the main products of *in vitro* metabolism of 1-(4-acetylphenyl)-3,3-dimethyltriazene are 1-(4-acetylphenyl-3-methyltriazene and 4-aminoacetophenone. In addition a novel metabolite, 1-[4-(1-hydroxyethyl)-phenyl]-3,3-dimethyltriazene, possessing antitumour activity similar to the parent drug, was identified.

INTRODUCTION

1-Aryl-3,3-dimethyl-triazenes are compounds which have shown antitumour activity in experimental animal tumours[1,2] and are possible candidates for clinical investigation in the near future. This class of compounds includes a number of analogues with different substituents in the 4-position of the aromatic ring. Interest in the metabolism of these compounds arises from the fact that they are inactive *per se* but require metabolic activation for activity.[3,4] In addition, there is still doubt as to whether different substituents influence the metabolism and consequently the pharmacological activity of these drugs.[5]

In this study, we use high-pressure liquid chromatography (HPLC) and mass spectrometry to confirm that 1(4-acetyphenyl)-3-methyltriazene (CH_3CO-MMT) and 4-amino-acetophenone (CH_3CO-PA) are the main products of *in vitro* metabolism of 1-(4-acetylphenyl)-3,3-dimethytriazene (CH_3CO-DMT) and report the identification of 1-[4-(1-hydroxyethyl)-phenyl]-3,3-dimethyl-triazene (CH_3CHOH-DMT) as a novel metabolite possessing antitumour activity similar to that of the parent drug.

EXPERIMENTAL

Drugs

1-(4-Acetylphenyl)-triazenes (CH_3CO-DMT, CH_3CO-MMT) were synthesized by the usual methods.[3] CH_3CHOH-DMT was prepared by lithium aluminium hydride reduction of CH_3CO-DMT.[6] CH_3CO-PA was purchased from BDH Chemicals Ltd.

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Abbreviations: CH_3CO-DMT = 1-(4-acetylphenyl)-3,3-dimethyltriazene; CH_3CHOH-DMT = 1-[4-(1-hydroxyethyl)phenyl]-3,3-dimethyltriazene; CH_3CO-PA = 4-amino-acetophenone; CH_3CO-MMT = 1(4-acetylphenyl)-3-methyltriazene; BSTFA = *N,O*-bis-(trimethylsilyl)-trifluoroacetamide; TMCS = trimethylchlorosilane, DMSO = dimethylsulfoxide; NADPH = β-nicotinamide-adenine dinucleotide phosphate, reduced; ADH = alchol dehydrogenase; NADH = β-nicotinamide-adenine dinucleotide, reduced.

In vitro metabolism

C57B1/6J female mice (20 ± 2 g body weight), obtained from Charles River Laboratories, Italy, were killed and the liver was immediately removed, weighed and homogenized in ice-cold 0.15 M potassium chloride buffer solution (pH = 7.4) in the ratio of 1 : 5 (w/v). The homogenate was centrifuged at 9000 × g for 20 min. Incubation was started by addition of substrate (CH_3CO-DMT) in acetone (0.1 ml) to the supernatant fraction (3 ml) with NADPH as cofactor.

Protein was determined by the method of Lowry *et al.*[7] using bovine serum albumin as a standard. CH_3CO-DMT concentration was 33 nmol ml^{-1}. Incubations were carried out at 37 °C in a shaken flask in a water bath in the presence of atmospheric oxygen, or under carbon monoxide to achieve anaerobic conditions. After 30 min, 1 ml aliquots of the incubation mixture were deproteinized by addition of ice-cold acetone (1 ml). Samples were injected into the high-pressure liquid chromatographic column after centrifugation at 600 × g for 3 min.

High-pressure liquid chromatographic analysis

CH_3CO-DMT and the metabolites were separated on a Waters chromatograph model 440 equipped with a 340 nm absorbance detector and a Lichrosorb RP18 column (Merck, Darmstadt, W. Germany). Separation of compounds was achieved with a linear gradient solvent system of aqueous acetonitrile containing diethylamine (0.01%). The initial composition of the mobile phase was 25% acetonitrile, the final (60% acetonitrile) being reached in 20 min with a flow rate of 1.7 ml min^{-1}. The eluates corresponding to CH_3CO-DMT and the metabolites were collected from the liquid chromatograph and extracted with hexane (5 volumes). After evaporation of the organic solvent, the dry residues were stored until mass spectral analysis. In order to obtain sufficient sample for mass spectral identification, the products of three incubation mixtures were processed in this manner and the eluates combined.

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MS analysis

An LKB 2091 mass spectrometer with an LKB 2130 computer was used for the gas chromatographic mass spectrometric analysis of CH_3CO-DMT and CH_3CO-MMT; for CH_3CO-PA and the unknown metabolites, the direct inlet system was used.

The gas chromatographic conditions were: column OV 1 3% on Gas Chrom Q 80–100 mesh, 2 m long, 2 mm i.d.; separator temperature 260 °C; electron impact energy 70 eV; helium carrier gas flow rate 25 ml min^{-1}. CH_3CO-MMT was identified as the trimethylsilyl derivative. This derivatization was carried out with BSTFA in pyridine in the presence of 1–2% TMCS as catalyst. All silylating agents were purchased from Pierce, Rockford, Illinois.

Antitumour activity

Mice received an i.m. transplant of 5×10^5 viable cells of M5076/73A ovarian reticular cell sarcoma, supplied by Mason Research Institute, DCT-Animal and Human Tumor Bank, Rochester, Massachussetts, USA. CH_3CO-DMT and CH_3COH-DMT were dissolved in a mixture of Tween 80, DMSO and saline (5 : 5 : 90) and injected i.p. at a dose of 20 mg kg^{-1} for 15 consecutive days starting on day 7 after tumour implantation. Control animals received the vehicle only. On day 25 after tumour implantation (the day corresponding to the median survival times of the controls), autopsies were carried out. Tumour, liver and ovaries were gossly inspected and weighed. Other groups of animals were used to evaluate the survival time as described by Geran *et al.*[8]

Enzymatic assay

CH_3CO-DMT was incubated with ADH from equine liver under the conditions described by Elliott *et al.*[9] The concentrations were: CH_3CO-DMT 100 μg ml^{-1}, ADH μg ml^{-1} and NADH 400 μg ml^{-1}. ADH and NADH were purchased from Boehringer, Mannheim, FRG. The final volume was 5 ml of 3 mM sodium phosphate buffer (pH = 7.0). The reaction was carried out at 24 °C in a shaken flask in a water bath exposed to the atmosphere. After 7 h, maximal formation of CH_3CHOH-DMT was observed and 1 ml of the incubation mixture was treated as described previously.

RESULTS AND DISCUSSION

Figure 1 shows the HPLC chromatogram of the metabolites produced from CH_3CO-DMT by incubation with a 9000 × g fraction of homogenized murine liver (a) without and (b) with cofactor: peak I corresponds to CH_3CO-PA, peak II to the unknown metabolite, peak III to CH_3CO-MMT and peak VI to CH_3CO-DMT. The mass spectrum of CH_3CO-DMT is shown in Fig. 2 and is that expected for a 1-aryl-3,3-dimethyltriazene.[10] The ion at m/z 191 is the molecular ion and loss of a methyl radical leads to the ion at m/z 176. The metastable ion at m/z 113.1 corresponds to the

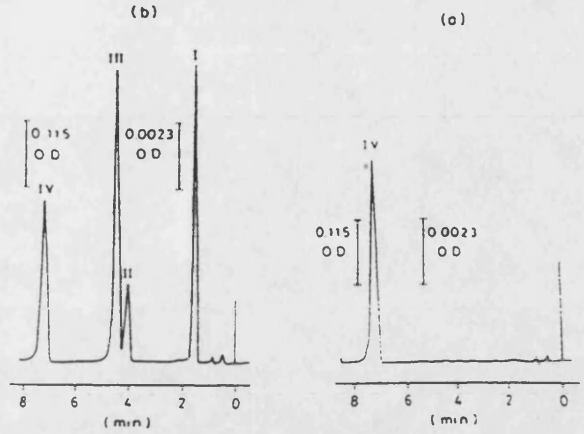

Figure 1. Chromatograms obtained injecting the deproteinized 9000 × g mouse liver mixture after 30 min incubation without (a) and with cofactor (b). Peak I corresponds to CH_3CO-PA, II to unknown compound, III to CH_3CO-MMT and IV to CH_3CO-DMT.

decomposition m/z 191 → m/z 147, with ejection of a dimethylamino radical, whereas the metastable ion at m/z 96.3 indicates the loss of 28 u (probably N_2, in analogy with the loss shown by CH_3CHOH-DMT, see later) from the ion at m/z 147. The resulting ion at m/z 119 is the most abundant peak. Further loss of 28 u (probably CO), evidenced by the metastable ion at m/z 69.6, yields the tropylium ion (m/z 91). All these structures are proposed on the basis of low resolution data, supported by the comparison between analogues (CH_3CO-DMT and CH_3-$CHOH$-DMT).

Figure 3(a) shows the mass spectrum of the compound corresponding to peak III of Figure 1(b) after silylation with BSTFA. This peak has the same HPLC retention time as authentic CH_3CO-MMT, as previously reported.[4] In Fig. 3(b), the mass spectrum of the trimethylsilyl derivative of authentic CH_3CO-MMT is shown and can be seen to be virtually identical to that in Fig. 3(a), thus confirming the previous assignment. In the mass spectrum of the trimethylsilylated CH_3CO-MMT, the molecular ion at m/z 249 is of low abundance, loss of 15 u (presumably CH_3) yields another low abundance fragment at m/z 234. The first major fragmentation of the molecular ion is, however, a loss of 42 u to give the ion corresponding to the large peak at m/z 207. This

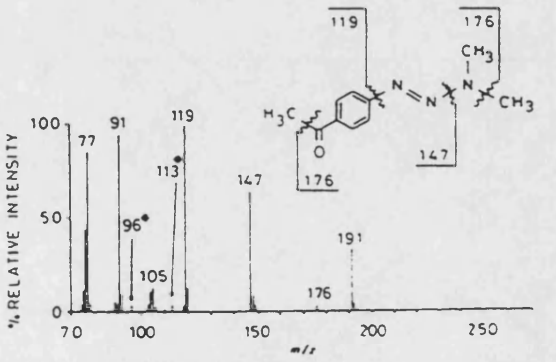

Figure 2. Electron impact mass spectrum of CH_3CO-DMT (IV) after HPLC collection.

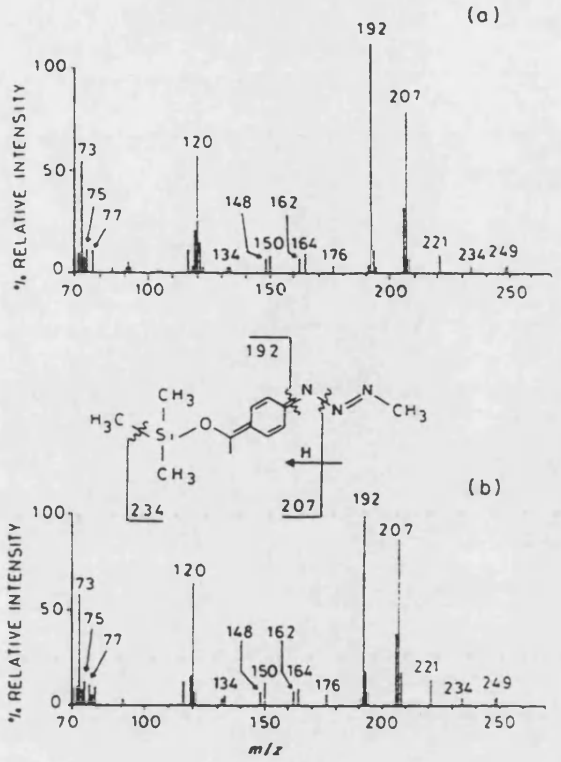

Figure 3. Electron impact mass spectrum of (a) component III as its trimethylsilyl derivative after HPLC collection; (b) CH_3CO-MMT standard as its trimethylsilyl derivative.

fragmentation has been suggested[10,11] to be characteristic of monoaryl monomethyl triazenes but not trisubstituted triazenes. It represents the loss of CH_2N_2 from the $ArNHNNCH_3$ tautomer giving the arylamine, thus suggesting that the silylation procedure has converted the acetyl moiety to the corresponding O-trimethylsilyl enol ether rather than effecting N-silylation at the triazene. The base peak, at m/z 192, can then be seen to correspond to loss of CH_3N_3 leading to the 1-phenyl-1-trimethylsilyloxy-ethene cation. Silicon-oxygen bond fission then furnishes the two remaining major fragment ions at m/z 120 with the transfer of H· and m/z 73 (Me_3Si^+).

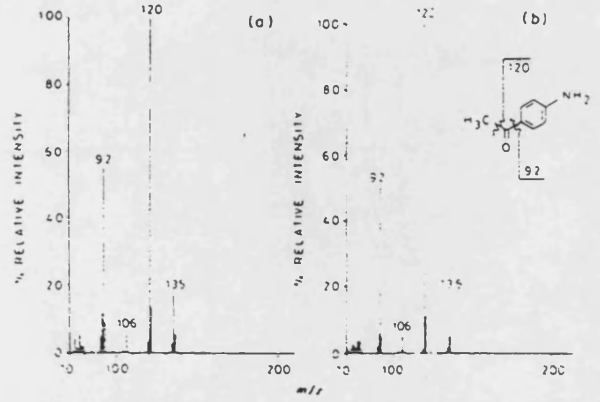

Figure 4. Electron impact mass spectrum of (a) component I (direct inlet system) after HPLC collection; (b) CH_3CO-PA standard (direct inlet system).

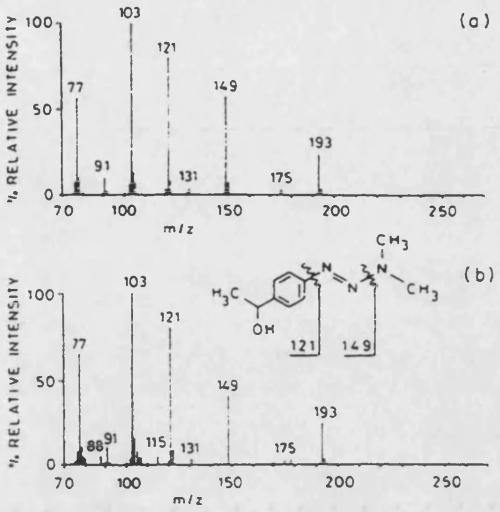

Figure 5. Electron impact mass spectrum of (a) component II (direct inlet system) after HPLC collection; (b) CH_3CHOH-DMT standard (direct inlet system).

Figure 4(a) shows the mass spectrum (direct insertion technique) of the compound corresponding to peak I of the HPLC chromatogram (Fig. 1(b)). We have previously reported[4] that this compound is CH_3CO-PA, on the basis of comparisons of HPLC retention time. The direct insertion mass spectrum of a commercial sample of CH_3CO-PA is shown in Fig. 4(b) and appears to be identical to that in Fig. 4(a), thus confirming our interpretation. The molecular ion is at m/z 135, loss of a methyl radical yields the most abundant peak at m/z 120, and loss of CH_3CO· gives the peak at m/z 92.

The unknown metabolite (peak II of the chromatogram in Fig. 1(b)) exhibited the direct insertion mass spectrum presented in Fig. 5(a). This spectrum contains, *inter alia*, a molecular ion at m/z 193 and abundant ions at m/z 149 and 121. These ions lie at

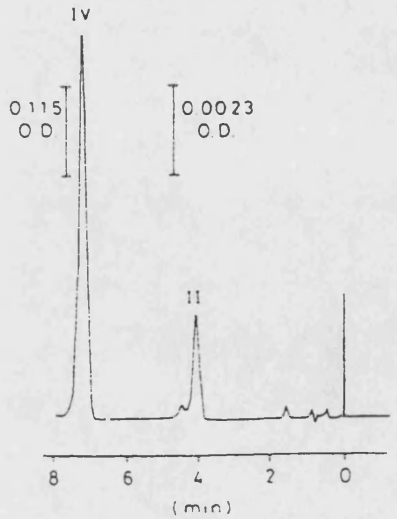

Figure 6. Chromatogram obtained injecting the deproteinized 9000 × g mouse liver mixture after 30 min incubation under CO flow. Peak II corresponds to unknown compound and peak IV to CH_3CO-DMT.

2 u greater than the corresponding ions derived from CH_3CO-DMT, suggesting that a modification to the aromatic ring or to the acetyl moiety but not to the triazene has taken place during metabolism. When the *in vitro* experiment was carried out in an atmosphere of carbon monoxide, thus inhibiting oxidative metabolism mediated by cytochrome P450, peak II was again evident in the HPLC trace whereas peaks I and III (arising from demethylated species) were absent (Fig. 6). This suggests that metabolism giving the compound of peak II is due to a reduction not involving haem centres such as cytochromes. This hypothesis is supported by evidence from the experiment in which CH_3CO-DMT is treated with a mixture of ADH and NADH which also gives a single product of HPLC characteristics identical to those of peak II. The identity of this hitherto-unknown was confirmed as CH_3CHOH-DMT by comparison with authentic material on the basis of HPLC retention time and mass spectrum. Figure 5(b) shows the mass spectrum of this synthetic CH_3CHOH-DMT with a prominent molecular ion at m/z 193. By comparing this spectrum with the spectrum of CH_3CO-DMT, we suggest that the fragmentation of CH_3CHOH-DMT occurs by two separate paths, one of high and one of low abundance. The major route involves the initial loss of 44 u. ($Me_2N^{\cdot}$) giving the 4-(1-hydroxyethyl)benzene diazonium ion (m/z 149), a process characteristic of 3,3-trimethyltriazenes.[10,11]

Loss of molecular nitrogen then yields an ion of m/z 121 which dehydrates to give the styryl carbonium ion at m/z 103. This ion is the point of convergence with the minor pathway in which the dehydration (to m/z 175) precedes the loss of Me_2N (to m/z 131) and N_2 (to m/z 103). Loss of acetylene from this base peak ion then furnishes the remaining ion of high abundance at m/z 77.

The antitumour activity of CH_3CHOH-DMT was found to be slightly greater than that of CH_3CO-DMT when tested against the murine ovarian reticular cell sarcoma M5076. The mass of the primary tumour was only slightly modified by treatment (3.9 ± 0.2 g for CH_3CO-DMT, 3.3 ± 0.2 g for CH_3CHOH-DMT and 4.2 ± 0.3 g for controls). Both showed clearcut antimetastatic effects in that none of the treated animals of each group showed evidence of metastases whereas all control animals presented metastatic involvement of liver and ovary. The median survival time of treated mice versus untreated controls $\times 100$ ($T/C \cdot 100$) was similar for both compounds: 141% for CH_3CO-DMT and 138% for CH_3CHOH-DMT.

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The Formation and Metabolism of *N*-Hydroxymethyl Compounds III - The Metabolic Conversion of *N*-Methyl and *N,N*-Dimethylbenzamides to *N*-Hydroxymethyl Compounds

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THE FORMATION AND METABOLISM OF *N*-HYDROXYMETHYL COMPOUNDS—III

THE METABOLIC CONVERSION OF *N*-METHYL AND *N,N*,-DIMETHYLBENZAMIDES TO *N*-HYDROXYMETHYL COMPOUNDS*

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Abstract—The stability of metabolically-generated *N*-(hydroxymethyl) compounds was investigated using a series of *N*-methylbenzamides as model substrates. *N*-(Hydroxymethyl)-benzamide was characterized as a major metabolite of *N*-methylbenzamide *in vitro*, and was also identified as a urinary metabolite of *N*-methylbenzamide. N-(Hydroxymethyl) compounds were also found as metabolites of 4-chloro-*N*-methylbenzamide and 4-*t*-butyl-*N*-methylbenzamide *in vitro*. Thus substitution in the 4-position of the phenyl ring of derivatives of *N*-(hydroxymethyl)-benzamide did not affect their stability sufficiently to cause degradation to formaldehyde under the conditions used. *N*-(Hydroxymethyl)-*N*-methylbenzamide was identified as a metabolite of *N,N*-dimethylbenzamide *in vitro*. However, *N*-(hydroxymethyl)-*N*-methylbenzamide was less stable than *N*-(hydroxymethyl)-benzamide under alkaline conditions. Furthermore, *N*-(hydroxymethyl)-*N*-methylbenzamide, unlike *N*-(hydroxymethyl)-benzamide and its 4-substituted derivatives, was positive in the colorimetric assay for formaldehyde, presumably because of its degradation to produce formaldehyde. Thus substitution on the nitrogen atom which bears the methyl group in *N*-methylbenzamide markedly affected the stability of the *N*-methylol produced during oxidative metabolism. *N*-Formylbenzamide was identified as a metabolite of *N*-methylbenzamide in suspensions of mouse hepatocytes and also *in vivo*. The mechanism for its production probably involves the generation of *N*-(hydroxymethyl)-benzamide.

N-(Hydroxymethyl) or *N*-methylol derivatives (**A**) of various amines and amides can be synthesized by reaction of the appropriate amine or amide with formaldehyde [1]. The reaction is readily reversible in aqueous media [2]. It is therefore not surprising that *N*-(hydroxymethyl) compounds produced during the oxidative *N*-demethylation of *N*-methyl-containing xenobiotics have been considered to be transient species which rapidly degrade to produce the *N*-desmethyl compound (**B**) and formaldehyde (**C**) [3, 4].

$$R_2N{-}CH_3 \xrightarrow{Fe^{3+}-[O]} \underset{\mathbf{A}}{R_2N{-}CH_2OH} \longrightarrow \underset{\mathbf{B}}{R_2NH} + \underset{\mathbf{C}}{CH_2O}$$

However, it was shown as early as 1953, in one of the first studies of metabolic *N*-demethylation *in vitro*, that the *N*-(hydroxymethyl) derivative of 3-methyl-4-methylaminoazobenzene had sufficient stability to undergo further chemical reaction with glutathione to form a methylene thioether [5]. Although this conclusion was tentative, there have since been many *N*-(hydroxymethyl) intermediates isolated during the metabolism of *N*-methyl-containing compounds. The evidence pertaining to the formation and properties of these intermediates has been recently summarized [6].

A study of the conditions under which *N*-(hydroxymethyl) compounds or alternatively formaldehyde are formed as metabolites of xenobiotics bearing *N*-methyl groups is toxicologically relevant, as such carbinolamines are capable of the aminomethylation of nucleophiles such as glutathione [7, 8] or may act as transport forms of formaldehyde [9], a carcinogen [10]. In an attempt to define those characteristics of a *N*-methyl-containing compound which predispose it to form a stable *N*-(hydroxymethyl) intermediate during metabolism, we have investigated the stability of both synthetic *N*-(hydroxymethyl) compounds and those produced during metabolism [6, 11–14]. Synthetic *N*-(hydroxymethyl) compounds have widely differing stabilities [15] but appear to be relatively stable when the *N*-(hydroxymethyl) group is in an electron-deficient environment [16–18]. An example of such a stable *N*-methylol is *N*-(hydroxymethyl)-

* For No. II in this series see [24].

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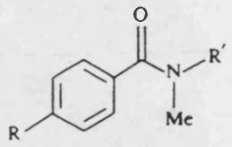
Abbreviations used: BSTFA, *N,O*-bis-(trimethylsilyl)-trifluorocetamide; HPLC: high pressure liquid chromatography, GC–MS: gas chromatography–mass spectrometry.



	R	R′
A	H	H
B	Cl	H
C	*t*-Bu	H
D	H	Me

Fig. 1. Derivatives of *N*-methylbenzamide used in this study.

benzamide, which can be readily synthesized [19] and has a half-life of 21.1 hr in Earl's buffer, pH 7.4, at 37° [6].

In this study we wished to test the hypothesis that the metabolism of *N*-methyl compounds which have the *N*-methyl moiety in an electron-deficient environment leads to stable *N*-(hydroxymethyl) derivatives and not to formaldehyde. As model compounds, we chose *N*-methylbenzamide [the methyl analogue of *N*-(hydroxymethyl)-benzamide], its 4-chloro and 4-*t*-butyl derivatives, and *N,N*-dimethylbenzamide (Fig. 1, **A–D**). These derivatives of *N*-methylbenzamide possess substituents which influence the electron density in the amide portion of the molecule [20], and thus may affect the stability of metabolites obtained by hydroxylation of the carbon atom of the *N*-methyl moiety.

MATERIALS AND METHODS

Chemicals. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase and NADP were purchased from Sigma Chemical Co. (Poole, U.K.). All *N*-methyl-substituted benzamides and their respective *N*-desmethyl derivatives were prepared by reaction of the appropriate benzoyl chloride with the corresponding amine by the Schotten–Baumann technique [21]. All *N*-(hydroxymethyl)-benzamides were prepared by treatment of the corresponding benzamide with excess aqueous formaldehyde (37%) in boiling tetrahydrofuran in the presence of potassium carbonate.

Animals. Male Balb c mice (20–25 g) were used for all experiments.

In vitro *metabolism.* Liver fractions and hepatocytes were prepared according to the methods described in [22] and [23], respectively. Incubations using liver fractions were performed in Earl's buffer, pH 7.4 (2.5 ml) in the presence of 5 mM $MgCl_2$ and sufficient glucose-6-phosphate, glucose-6-phosphate dehydrogenase and NADP to yield 1 mM NADPH. Reactions were started by the addition of substrate in a volume of not more than 0.1 ml of the solvent indicated below. Substrate concentrations used were: *N*-methylbenzamides 10 mM (methanol), except in the case of incubations with isolated hepatocytes where a concentration of 1 mM (acetone) was used: *N,N*-dimethylbenzamide 5 mM (acetone). Microsomes were added to give concentrations equivalent to 1 g and 0.7 g wet liver weight/2.5 ml in the case of incubations containing *N*-methylbenzamide and *N,N*-dimethylbenzamide, respectively. Supernatant (9000 g) was added to give a concentration equivalent to 0.25 g wet liver weight/2.5 ml. Incubations with hepatocytes were performed as described previously [24].

At the end of the incubation period, reactions were stopped by immersing the incubation mixtures in ice. For HPLC analysis 0.15 ml of the appropriate internal standard (see below) was added and the mixtures were prepared as described previously [24]. For colorimetric analysis of formaldehyde equivalents formed during microsomal metabolism, microsomes were removed by centrifugation at 27,000 *g* for 20 min. Then to 2 ml of the supernatant from duplicate incubations, either 0.25 ml Earl's buffer, pH 7.4, or 0.25 ml 1 M NaOH (to hydrolyse stable carbinolamines) was added. The mixtures were left at room temperature for 5 min and were then assayed for formaldehyde according to the method of Nash [25].

In vivo *metabolism.* *N*-Methylbenzamide was injected into mice i.p. in 0.1 ml of 10% DMSO/arachis oil to give a dose of 200 mg/kg. Urine was collected and prepared for analysis as described previously [24].

HPLC analysis. Separation of metabolites was performed using an Altex 100A pump, a Waters radial compression unit equipped with a C_{18}, 5 μm reverse phase column and a Pye LC–UV detector (λ = 254 nm). The conditions used for the separation of standard compounds were: (a) *N,N*-dimethylbenzamide, *N*-methylbenzamide, *N*-(hydroxymethyl)-benzamide and benzamide—mobile phase 30% methanol–water, flow rate 2 ml/min, internal standard 4-nitrobenzamide 0.2 mg/ml in methanol; (b) 4-chloro-*N*-methylbenzamide, 4-chloro-*N*-(hydroxymethyl)-benzamide and 4-chlorobenzamide—mobile phase 50% methanol–water, flow rate 1.5 ml/min, internal standard 4-nitrobenzamide 0.2 mg ml in methanol; (c) 4-*t*-butyl-*N*-methylbenzamide, 4-*t*-butyl-*N*-(hydroxymethyl)-benzamide and 4-*t*-butylbenzamide—mobile phase 57% methanol–water, flow rate 2 ml/min, internal standard 4-chlorobenzamide 0.2 mg/ml in methanol.

Isolation and characterization of metabolites. HPLC eluates corresponding to peaks with the retention times of authentic standards were collected from the column and evaporated to dryness under a stream of nitrogen at room temperature. The residues were dissolved in acetone (0.025 ml) and subjected to mass spectral analysis. Samples were either admitted directly to the mass spectrometer under chemical ionization conditions (as described previously [24]) or where derivatized and analyzed by electron impact GC–MS.

Derivatization of isolated metabolites. Metabolites and standard compounds, chromatographically isolated, were dissolved in pyridine (0.05 ml), and BSTFA (0.05 ml) was added. The mixtures were heated at 60° for 15 min and subjected to GC–MS analysis.

GC–MS analysis. GC–MS analysis was performed

Fig. 2. High pressure liquid chromatograms of extracts of (a) a mixture of the following reference compounds: 1, *N*-hydroxymethylbenzamide; 2, benzamide; 3, internal standard; 4, *N*-methylbenzamide; (b) a mixture of *N*-methylbenzamide (10 mM) with microsomes and a NADPH-generating system, incubated for 30 min at 37°; and (c) as incubation mixture (b) but omitting *N*-methylbenzamide.

using a Pye 204 gas chromatograph equipped with a fused silica SE52 capillary column linked to a VG 7070 mass spectrometer. The injector temperature was 250°, the column temperature was 100° initially increasing by 32°/min for 5 min. A 1:10 split of carrier gas (helium) was used, the mass spectrometer source temperature was 220°, and an electron energy of 70 eV was used.

RESULTS

The incubation of *N*-methylbenzamide with microsomes and an NADPH-generating system produced a metabolite with a retention time identical to that of *N*-(hydroxymethyl)-benzamide (1, Fig. 2). The identity of this metabolite was confirmed by comparison with authentic *N*-(hydroxymethyl)-benzamide using GC–MS analysis after derivatization with BSTFA (Fig. 3). 4-Chloro-*N*-methylbenzamide and 4-*t*-butyl-*N*-methylbenzamide were likewise metabolized to their respective *N*-(hydroxymethyl) derivatives in microsomes. These metabolites were characterized by comparison of their retention times with those of authentic standards and, in the case of 4-chloro-*N*-(hydroxymethyl)-benzamide, mass spectrometry (not shown). The extent of biotransformation of *N*-methylbenzamide and its 4-chloro and 4-*t*-butyl derivatives to their respective *N*-methylols and *N*-desmethyl derivatives in microsomal and 9000 *g* supernatant fractions of liver is shown in Table 1.

A chromatogram of an extract of the products of metabolism of *N*-methylbenzamide by isolated hepatocytes is shown in Fig. 4. *N*-(hydroxymethyl)-benzamide (1, Fig. 4), benzamide (2, Fig. 4) and *N*-formylbenzamide (5, Fig. 4) were identified as metabolites of *N*-methylbenzamide (4, Fig. 4) on the basis of their chromatographic retention times. These metabolites were also detected in the urine of mice which had received *N*-methylbenzamide, 200 mg/kg i.p. (Fig. 5). 4-Chloro-*N*-methylbenzamide and 4-*t*-butyl-*N*-methylbenazmide were also metabolized to their *N*-(hydroxymethyl) derivatives by isolated hepatocytes and the extent of their biotransformation compared to that of *N*-methylbenzamide is shown in Table 2.

On incubation with microsomes, *N*,*N*,-dimethylbenzamide (4, Fig. 6) produced a metabolite (3, Fig. 6) with a retention time identical to that of *N*-methylbenzamide and another metabolite (1, Fig. 6). The identity of metabolite 3 (Fig. 6) was confirmed as *N*-methylbenzamide by mass spectrometry. Metabolite 1 (Fig. 6) decomposed upon alkaline hydrolysis with a corresponding increase in the amount of *N*-methylbenzamide present in the mixture. The time course of production of metabolites 1 and 3 (Fig. 6) is shown in Fig. 7, and indicates that the amount of metabolite 1 first increased and then decreased. The decrease was presumably due to chemical degradation or further metabolism of metabolite 1. Metabolite 1 was derivatized using BSTFA and subjected to GC–MS analysis. The mass spectrum obtained indicated that metabolite 1 was *N*-(hydroxymethyl)-*N*-methylbenzamide [*m/z* 237, M^+ for $C_6H_5CON(CH_3)CH_2OSi(CH_3)_3$, 61.2%; *m/z* 222 $[C_6H_5CON(CH_3)CH_2OSi(CH_3)_2]^+$, 37.5%; *m/z* 192 $[C_6H_5C(OSi(CH_3)_2)=NCH_3]^+$, 40.7%; *m/z* 105, $C_6H_5CO^+$, 100%]. Attempts to synthesize *N*-hydroxymethyl-*N*-methylbenzamide were not successful.

Formaldehyde, or a precursor which reacted like formaldehyde with Nash reagent, was generated during the microsomal metabolism of *N*,*N*-dimethylbenzamide and the amount of Nash-positive species in the incubation mixture increased after alkaline hydrolysis (Table 3). In the case of *N*-methylbenzamide no formaldehyde could be detected in the metabolism mixture unless alkaline hydrolysis was

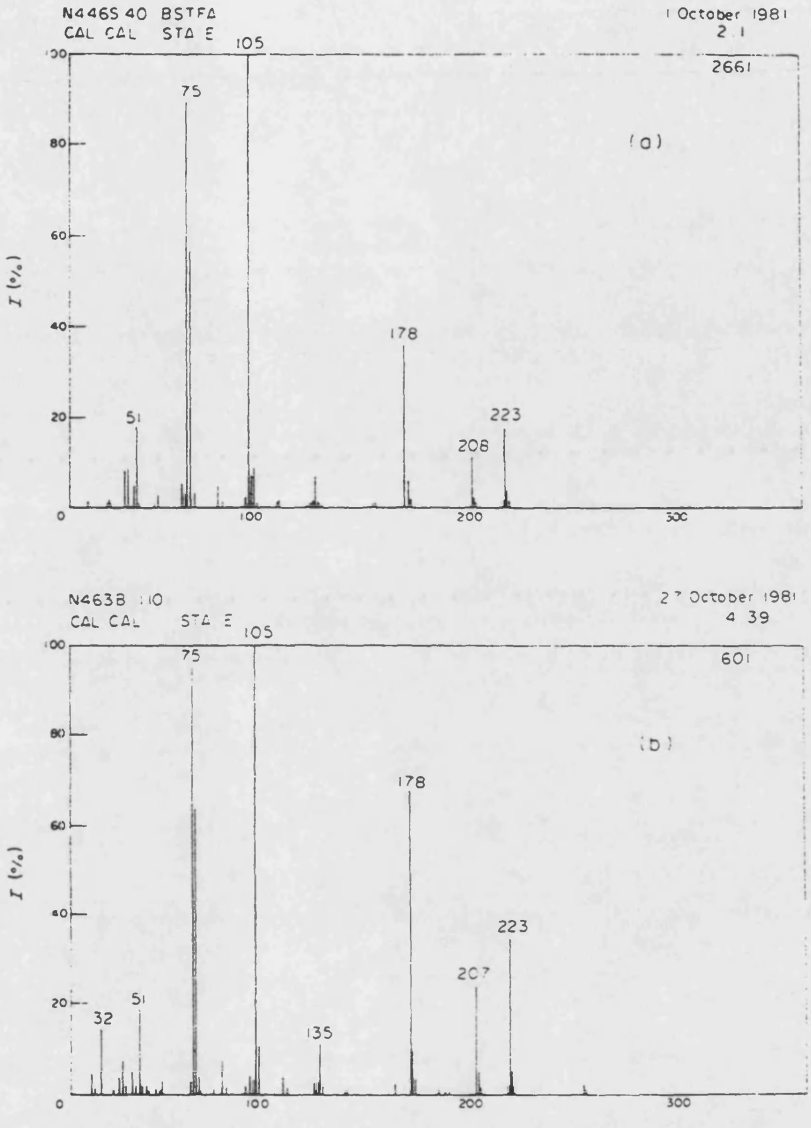


Fig. 3. Mass spectra of a peak (Rt = 8.8 min) produced during the GC–MS analysis of authentic *N*-hydroxymethylbenzamide (a) and an *in vitro* metabolite of *N*-methylbenzamide with an identical retention time on HPLC (b), after derivatization with BSTFA.

Table 1. The extent of *N*-demethylation of 4-substituted *N*-methylbenzamides (10 mM) in microsomal and 9000 *g* supernatant fractions of liver

	% Metabolism* to			
	N-(hydroxymethyl) derivative		*N*-desmethyl derivative	
Substrate	microsomes	9000 *g* supernatant	microsomes	9000 *g* supernatant
N-Methylbenzamide	2.8	2.9	0	0
4-Chloro-*N*-methylbenzamide	1.7	1.7	0	0
4-*t*-Butyl-*N*-methylbenzamide	0.6	0.5	0	0.2

* Mixtures contained either microsomes equivalent to 1 g wet liver weight or 9000 *g* supernatant equivalent to 0.25 g wet liver weight and sufficient cofactors to generate 1 mM NADPH. Mixtures were incubated at 37° for 30 min.

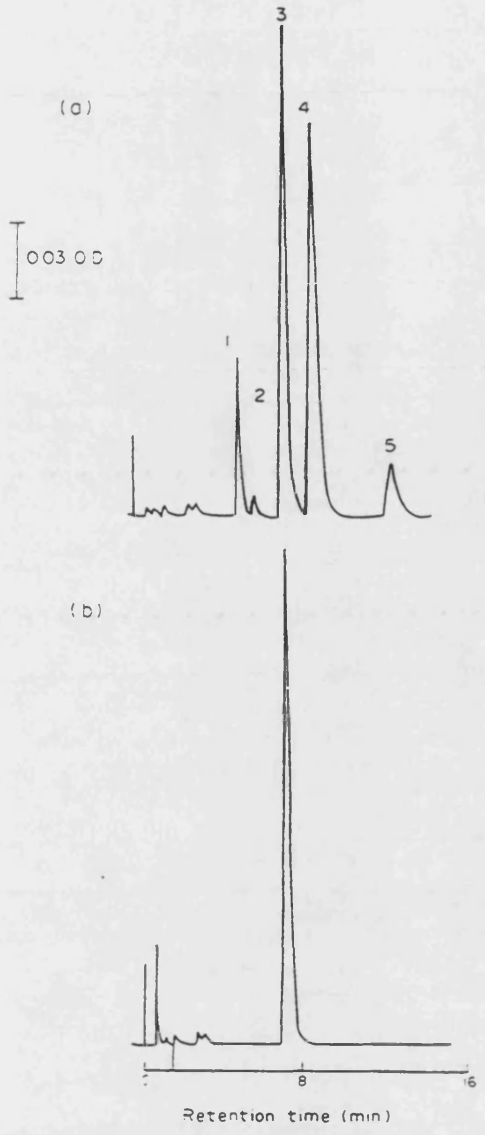


Fig. 4. High pressure liquid chromatograms of (a) an extract of a mixture of *N*-methylbenzamide (1 mM) with hepatocytes incubated at 37° for 90 min; and (b) an extract of an incubation of mixture omitting *N*-methylbenzamide. (1, *N*-hydroxymethylbenzamide; 2, benzamide; 3, internal standard; 4, *N*-methylbenzamide; 5, *N*-formylbenzamide).

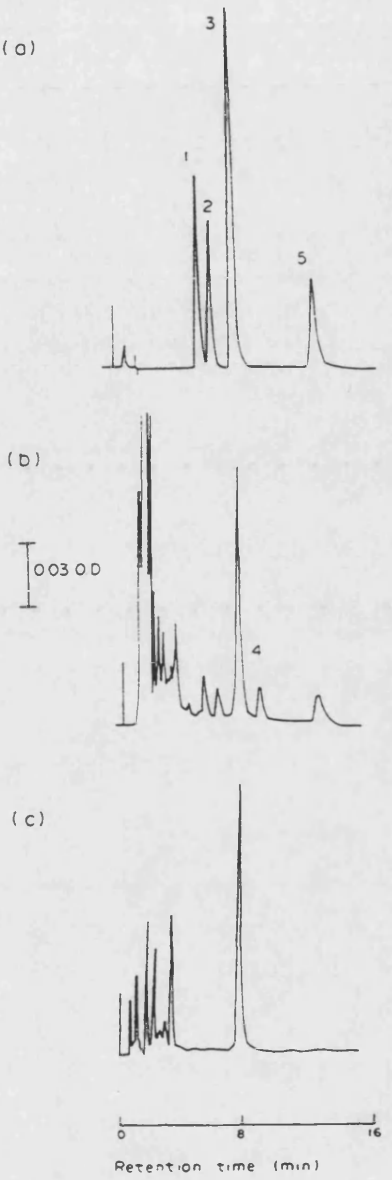


Fig. 5. High pressure liquid chromatograms of extracts of (a) a mixture of reference compounds (for identification see legend to Fig. 4); (b) urine from mice which had received *N*-methylbenzamide (4) 200 mg/kg i.p.; and (c) urine from mice which had received vehicle only.

Table 2. The metabolism of 4-substituted *N*-methylbenzamides (1 mM) by isolated hepatocytes

Substrate	% Metabolism* to *N*-(hydroxymethyl) derivative	*N*-desmethyl derivative	*N*-formyl derivative
N-Methylbenzamide	10.5	2.5	2.2
4-Chloro-*N*-methylbenzamide	8.6	6.9	n.d.
4-*t*-Butyl-*N*-methylbenzamide	∓	∓	n.d.

* After 90 min incubation at 37° with 5×10^5 cells/ml.
∓ Below 0.1%.
n.d., Not determined.

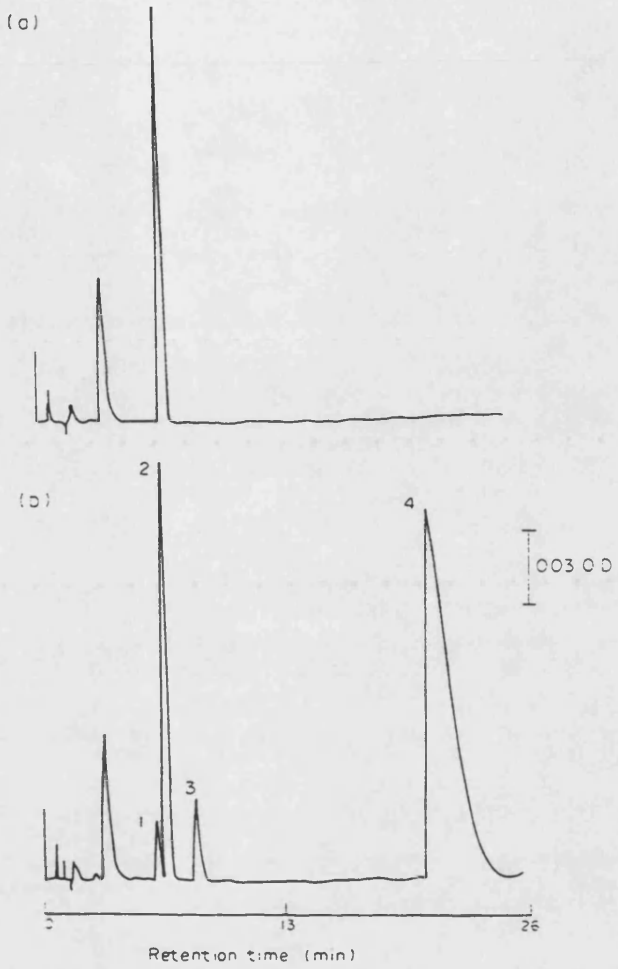


Fig. 6. High pressure liquid chromatograms of (a) an extract of a mixture of microsomes and a NADPH-generating system incubated for 30 min at 37°; (b) an extract of an identical incubation mixture containing *N,N*-dimethylbenzamide (5 mM). (1, Unidentified metabolite; 2, internal standard; 3, *N*-methylbenzamide; 4, *N,N*-dimethylbenzamide.)

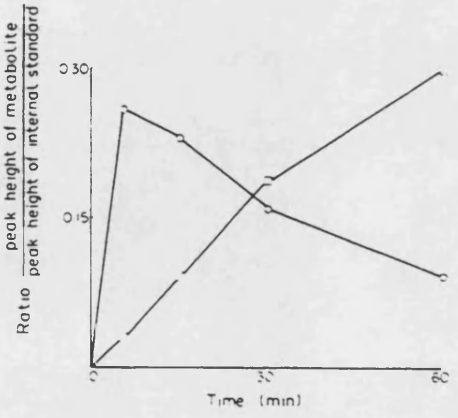


Fig. 7. Time course of production of *N*-methylbenzamide (□) and an unidentified metabolite (○) during the metabolism of *N,N*-dimethylbenzamide (5 mM) in microsomes.

performed prior to the Nash reaction (Table 3). It was conceivable that the differences in the production of Nash-positive species during the metabolism of *N*-methylbenzamide and *N,N*-dimethylbenzamide were due to the different stabilities of their respective *N*-(hydroxymethyl) compounds formed during metabolism. This was confirmed by an investigation of the stability of authentic *N*-(hydroxymethyl)-benzamide and metabolically-generated *N*-(hydroxymethyl)-*N*-methylbenzamide in various buffer systems at 37° (Fig. 8). The results show that under alkaline conditions *N*-(hydroxymethyl)-benzamide is more stable than *N*-(hydroxymethyl)-*N*-methylbenzamide.

In all *in vitro* incubations, no other metabolites were identified when the pH of the incubation mixtures were adjusted to pH 2 and re-extracted after the normal extraction process had been performed. This implies the absence of phenolic metabolites which may have been ionized at pH 7.4 and thus

Table 3. Formaldehyde equivalents generated during the microsomal metabolism of *N,N*-dimethylbenzamide (5 mM) and *N*-methylbenzamide (10 mM), measured using the Nash reagent before and after alkaline hydrolysis

Substrate	Formaldehyde equivalents (μM) measured Before alkaline hydrolysis	After alkaline hydrolysis
N,N-dimethylbenzamide (5 mM)	195	349
N-Methylbenzamide (10 mM)	0	90

Results are from one experiment, representative of three.

may not have been removed during the first extraction. When the pH of the urine of mice which had received *N*-methylbenzamide was adjusted to pH 2 and re-extracted, the liquid chromatogram of the extract showed two major peaks which were not extracted from control urine adjusted to pH 2. The chemical ionization mass spectrum of one of these metabolites suggested that it may be hippuric acid [*m/z* 180, MH^+, 100%; *m/z* 135, $(MH-COOH)^+$, 13%; *m/z* 105, $C_6H_5CO^+$ 66%]. Benzoic acid can be converted to hippuric acid in animals [26] and thus it is possible that this metabolite was formed *in vivo* by enzymic hydrolysis of the amide moiety followed by conjugation with endogenous glycine.

DISCUSSION

Although there are many examples in the literature of the biotransformation of *N*-methyl-containing xenobiotics to characterizable metabolites which possess *N*-(hydroxymethyl) groups (summarized in [6]), the conditions and structural requirements for their formation have not been studied. We report here that *N*-(hydroxymethyl)-benzamide, a stable compound which can be readily synthesized from formaldehyde and benzamide, is indeed a major metabolite *in vitro* and a minor metabolite *in vivo* of *N*-methylbenzamide. 4-Substitution in the phenyl ring of *N*-(hydroxymethyl)-benzamide alters its rate of breakdown to produce formaldehyde [6, 19]. However, 4-chloro-*N*-(hydroxymethyl)-benzamide and its 4-*t*-butyl derivatives both have half-lives in Earl's buffer, pH 7.4, at 37° of greater than 9 hr [6]. Thus these compounds would be expected to be sufficiently stable to be isolated using the conditions described in this study. This was confirmed by the characterization of *N*-methylols during the metabolism *in vitro* of 4-chloro-*N*-methylbenzamide and its 4-*t*-butyl derivative. Therefore although 4-substitution in the phenyl ring of *N*-methylbenzamides does result in quantitative differences in their metabolism (Tables 1 and 2), it does not alter the stability of the respective *N*-methylol derivatives sufficiently to cause qualitative differences in metabolism, i.e. to affect whether a stable *N*-(hydroxymethyl) compound or formaldehyde is produced.

N-(Hydroxymethyl)-benzamide and its 4-chloro and 5-*t*-butyl derivatives, like *N*-(hydroxymethyl)-formamide [15], are so stable at pH 7.4 that they do

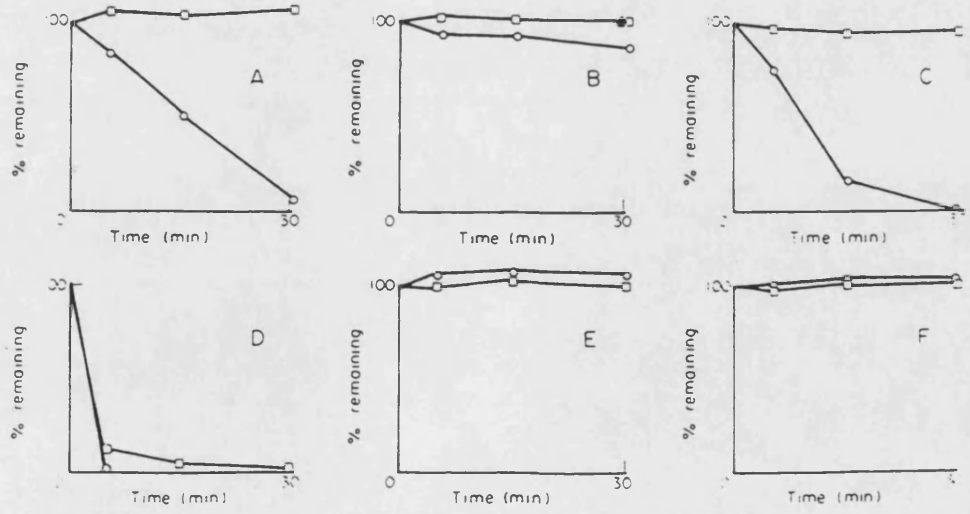


Fig. 8. The stability of authentic *N*-(hydroxymethyl)-benzamide (□) and metabolically-generated *N*-(hydroxymethyl)-*N*-methylbenzamide. (○) at 37° in the following buffer system: A—Earl's, pH 7.4; B—0.01 M Tris, pH 7.4; C—0.01 M Tris, pH 9; D—0.01 Tris, pH 12; E—acetate, pH 5; F—acetate, pH 2.8.

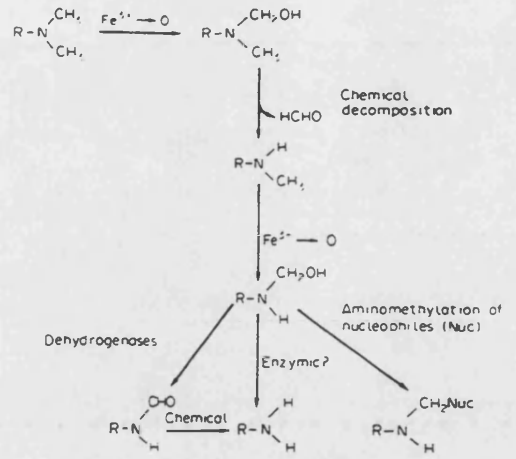
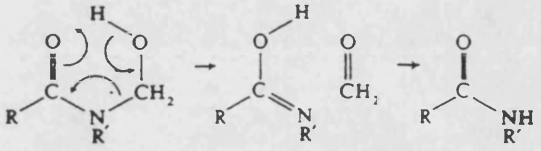


Fig. 9. Proposed scheme for the metabolism of the methyl groups of *N*-methyl-substituted benzamides.

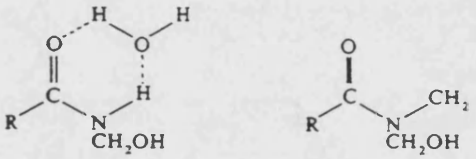
not react in the Nash assay. This assay detects formaldehyde and is used routinely to measure metabolic *N*-demethylation. Thus, assessment of the *N*-demethylation of these compounds by means of the Nash reaction, without a confirmatory chromatographic analysis, would produce erroneous results.

N-Formylbenzamide (5, Fig. 4 and 5), as well as *N*-(hydroxymethyl)-benzamide (1, Figs, 4 and 5) and benzamide (2, Figs. 4 and 5), were identified as metabolites of *N*-methylbenzamide (4, Figs. 4 and 5) in hepatocytes and *in vivo* on the basis of chromatographic retention times. We have shown previously that *N*-(hydroxymethyl)-benzamide can be metabolized *in vitro* to *N*-formylbenzamide by pyrazole-sensitive dehydrogenases [24]. Unlike *N*-(hydroxymethyl)-benzamide, *N*-formylbenzamide is an unstable species and degrades rapidly to produce benzamide. Thus it seems probable that the route whereby *N*-formylbenzamide and benzamide are produced *in vivo* involves the production and further metabolism of *N*-(hydroxymethyl)-benzamide (see Fig. 9).

Surprisingly, another substituent on the nitrogen atom of *N*-methylbenzamide markedly affected the stability of the *N*-methylol produced during *N*-demethylation. The *N*-(hydroxymethyl) compound produced during the metabolism of *N,N*-dimethylbenzamide was an unstable species and degraded partly to produce formaldehyde under the conditions used for *in vitro* metabolism studies. *N*-(Hydroxymethyl)-*N*-methylbenzamide, unlike *N*-(hydroxymethyl)-benzamide, produced a positive response in the Nash test prior to its hydrolysis by alkali (Table 3). Therefore, in the metabolic scheme proposed for the metabolism of the *N*-methyl groups in *N,N*-dimethylbenzamide (Fig. 9), the first *N*-demethylation leads to formaldehyde liberated from the degradation of an unstable *N*-methylol whereas the second *N*-demethylation yields a stable *N*-methylol. That the *N*-(hydroxymethyl)-*N*-methyl derivative is less stable than the *N*-(hydroxymethyl) derivative may be due to the electron-donating effect of the second methyl moiety which would oppose the electron-withdrawing effect of the carbonyl group. Alternatively the lack of an amide hydrogen atom in *N*-(hydroxymethyl)-*N*-methylbenzamide may prevent solvent participation in hydrogen bonding and this may account for the difference in stabilities. Tanka *et al.* [27] have suggested that intramolecular hydrogen bonding of *N*-(hydroxymethyl)-*N*-phenylureas (R = C_6H_5NH) leads to their degradation as described below and this may also apply to *N*-(hydroxymethyl)-benzamides (R = C_6H_5).



These workers suggested that the greater stability of the *N*-(hydroxymethyl) derivative (I) as opposed to the *N*-(hydroxymethyl)-*N*-methyl derivative (II) may be due to the fact that the *N*-hydroxymethyl group in I can be held in a configuration *trans* to the carbonyl group by a water molecule.



I II

In the case of II this hydrogen bonding is not possible due to the lack of an amide hydrogen atom, and consequently the formation of the cyclic transition state leading to breakdown of the molecule is more probable. Another possibility is that lack of the amide hydrogen atom in II may prevent tautomerism to the enol form of the molecule, which would prevent the generation of the transition state and subsequent degradation. Whatever the reason, it is conceivable that *N*-methylols of different stabilities may be generated during the successive *N*-demethylations of other *N*-methyl-containing substrates.

Like *N*-methylols produced during successive *N*-demethylations of the same substrate, *N*-methylols generated during the metabolism of different types of *N*-methyl-containing compound may have varying stabilities [15]. Furthermore, different types of *N*-methylols may undergo degradation by different pH-dependent mechanisms. For example, it has been shown that *N*-methylols derived from *N*-methylmelamines are more stable in basic rather than acidic solution [28], whereas *N*-methylolamides such as *N*-(hydroxymethyl)-benzamide [6, 19] and *N*-(hydroxymethyl)-nitrosamines [29] are more stable under acidic conditions. This varying stability of *N*-methylols may have toxicological implications as either the intact *N*-methylol [7, 8] or its degradation products, formaldehyde [2] and in certain cases the *N*-des-(hydroxymethyl) species [30–32], are capable of reacting with biological nucleophiles. It is therefore conceivable that particular types of *N*-methyl may be responsible for the antitumour activity observed with certain *N*-methyl-containing com-

pounds, e.g. *N*-methylmelamines [33], 1-aryl-3,3-dimethyltriazenes [34] and *N*-methylformamide [35]. Similarly the generation of *N*-methylols of a particular stability or reactivity may contribute to the toxicity and carcinogenicity of other *N*-methyl-containing compounds such as *N,N*-dimethylnitrosamine [36], azoxymethane [37] and *N,N*-dimethylaminoazobenzene [38]. This hypothesis and the possible involvement of *N*-formyl compounds produced during *N*-demethylation in the pharmacological and toxicological effects of *N*-methyl-containing compounds are currently under investigation in these laboratories.

Acknowledgements—We thank the Medical Research Council for financial support and J. Lamb for expert technical assistance.

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PUBLICATION 6

Labelled Compounds of Interest as Antitumour Agents I -
N-Methylformamide and *N,N*-Dimethylformamide

M. D. Threadgill and E. N. Gate

Journal of Labelled Compounds and Radiopharmaceuticals, **1983**, *20*, 447-451.

LABELLED COMPOUNDS OF INTEREST AS ANTITUMOUR AGENTS I: N-METHYLFORMAMIDE AND N,N-DIMETHYLFORMAMIDE.

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SUMMARY

Efficient preparations of N-methylformamide (an active antitumour agent) have been developed in which the compound is labelled with ^{14}C in the formyl group, with ^{14}C in the methyl group and with deuterium in the methyl group. N,N-Dimethyl-^{14}C-formamide has also been synthesised.

Key words: Antitumour, Carbon-14, Deuterium, Dimethylformamide, Methylformamide

INTRODUCTION

N-Methylformamide (1; NMF) is a compound which shows significant activity against a number of murine tumours [1-4] whereas that of its dimethyl analogue N,N-dimethylformamide (2; DMF) is, at best, marginal. In order to investigate both the *in vivo* distribution and the metabolic fate of the two different one-carbon fragments arising from NMF and DMF, we required NMF labelled with ^{14}C in each of the carbon positions separately (3 and 4) and DMF containing a ^{14}C methyl group (5). As it is possible that *in vivo* oxidation of the methyl group of NMF is responsible for furnishing the actual antitumour agent, we sought N-(trideutero methyl)-formamide (6) as a tool to examine any primary kinetic isotope effect in this putative metabolic activation; if such an effect were found, it may manifest itself, *inter alia*, as a decrease in antitumour activity upon deuteration.

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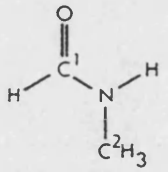
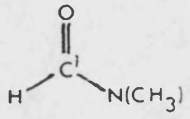
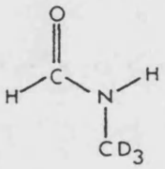


1: $C^1=C^2=\ ^{12}C$
3: $C^1=\ ^{12}C:C^2=\ ^{14}C$
4: $C^1=\ ^{14}C:C^2=\ ^{12}C$



2: $C^1=\ ^{12}C$
5: $C^1=\ ^{14}C$



6

RESULTS

The pyrolysis of ammonium salts of carboxylic acids has long been known to give carboxamides, albeit infrequently in modest yield. The synthesis of N,N-dimethyl-^{14}C-formamide (5) by this route has been described by Veres et al [5]: however, in our hands, in experiments using unlabelled material, this technique failed, giving a mixture of DMF (2) and dimethylammonium formate. A modification was therefore employed. Sodium ^{14}C-formate was heated with an excess of methylamine hydrochloride in the absence of solvent in an apparatus designed to permit gentle reflux before allowing the product to distil. This served to ensure that all the volatile methylammonium ^{14}C-formate had been thermally converted to the desired amide (3). The dimethyl analogue (5) was prepared similarly from sodium ^{14}C-formate and dimethylamine hydrochloride.

The methyl-radiolabelled compound (4) was prepared from ^{14}C-methylamine hydrochloride and ethyl formate in the presence of methanolic base. Chemical

and radiochemical yields of compounds (3 - 5) were high.

Pure anhydrous N-(trideuteromethyl)-formamide (6) was prepared from trideuteromethylamine hydrochloride and ethyl formate in moderate yield. All the labelled NMF derivatives (3, 4, 6) are new compounds. Differences between the infra-red spectrum of (6) and that of its protio-analogue (1) permitted us to assign certain absorbance bands to vibrations of the methyl group of NMF ($\nu_{C-H}/\nu_{C-D} \sim 1.37$). These assignments complement the work of Suzuki [6], who used other deuterated NMF derivatives, but conflict with Sutherland's [7] proposals for assignment of the bands at 2880 cm^{-1} and 960 cm^{-1} in the spectrum of (1), which we have shown to be vibrations of the CH_3 group (see the Table).

Table. Infra-red Spectrum of 1 and 6

ν (1)	ν (6)	ν(1)/ν (6)	Assignment
3400	3500-3200	1	NH
3060	3040	1.01	CH
3060	2230	1.37	CH_3
2950	2110	1.40	CH_3
2880	2080	1.38	CH_3
1660	1660	1.00	Amide I
1550	1520	1.02	Amide II
1420	1050	1.35	CH_3
1390	1385	1.00	CH
1240	1250	0.99	Amide III
1150	870	1.32	CH_3
960	700	1.37	CH_3

The outcome of the metabolic and pharmacokinetic investigations will be published elsewhere [8]; further studies on the physicochemical properties of this important antitumour agent (NMF, 1) are being actively pursued.

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Experimental

The radioactivity was measured by the scintillation method using a Packard Tricarb 2660 intrument and radiochemical purity was checked using thin-layer chromatography on silica gel plates (Silica Gel 60 F_{254}, Merck). Infrared spectra were obtained from thin films of liquid sample using a Perkin-Elmer 1310 spectrometer. Proton magnetic resonance spectra were obtained at 60 MHz using a Varian EM-360A spectrometer with tetramethylsilane as internal standard. Radiochemicals (^{14}C-methylamine hydrochloride and sodium ^{14}C-formate) were obtained from Amersham International PLC and trideuteromethylamine hydrochloride (98%D) from Aldrich Chemical Co.Ltd.

N-^{14}C-Methylformamide (3)

A solution of sodium methoxide in methanol (2.0ml) (prepared from sodium metal (69 mg)) was added slowly to a cooled solution of ^{14}C-methylamine hydrochloride (202.5 mg; 1.0 mCi) in methanol (1.0 ml). Anhydrous sodium carbonate (318 mg) and ethyl formate (3.0 ml) were added and the mixture was stirred at 25°C for 18 hours. Ethyl formate (4.0 ml) was added and the salts were filtered off using a cotton-wool plug. The filtrate and ethyl formate washings were combined and the solvents evaporated carefully under reduced pressure to give N-^{14}C-methylformamide (163 mg; 0.92m Ci; chemical yield 92%) as a colourless liquid. It had chromatographic properties identical to a commercial sample of unlabelled N-methylformamide.

N-Methyl-^{14}C-formamide (4)

Methylamine hydrochloride (270 mg) and sodium ^{14}C-formate (136 mg; 1.7m Ci) were placed in the side-arm of a 7cm x 1cm test-tube equipped with 10cm x 1cm side-arm and with a reflux condenser. Gentle heating of the solids over a flame for 8 minutes caused the volatile materials to boil under reflux within the side-arm. The mixture was then heated more strongly and a pale straw-coloured liquid distilled into the main tube. The distillate was extracted with acetone (6x4 ml) Careful evaporation of the solvent furnished N-methyl-^{14}C-formamide monohydrate (122 mg; 1.35 mCi; chemical yield 79%) as a pale yellow liquid. It had properties identical tc an equimolar mixture of authentic N-methylformamide and water.

N,N-Dimethyl-^{14}C-formamide (5)

This compound was prepared using a technique similar to that used for (4) from dimethylamine hydrochloride (326 mg) and sodium ^{14}C-formate (136 mg; 870 µCi) N,N-Dimethyl ^{14}C-formamide monohydrate (120 mg; 560µCi; chemical yield 66%) was obtained as a pale straw-coloured liquid having properties identical to an equimolar mixture of authentic N,N-dimethylformamide and water.

N-(Trideuteromethyl)-formamide (6)

Sodium (660 mg) was added to absolute ethanol (30 ml) at -5°C. When all the metal had dissolved, (trideuteromethyl)amine hydrochloride (2.0g) was added, followed by ethyl formate (25 ml) and anhydrous sodium carbonate (10g). The mixture was stirred at -5°C for 1 hour then 12 hours at 20°C. A clear solution was obtained after filtration and the filtrate and ethyl formate washings were concentrated in vacuo to 10ml. Distillation using the bulb-to-bulb technique gave N-(trideuteromethyl)-formamide (860 mg; 49%) a colourless liquid. BPt_3 65-70°C ν_{max} (liquid film): (see Table). $\delta(CDCl_3)$: 7.3 (1H) br (NH, 8.03) 0.1H) s CHO cis-form, 8.27 (0.9H) s CHO trans-form. m/z: 62 (M^+).

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PUBLICATION 7

Selective Reactions in the Triazene Series I -
Reduction of 1-(4-Acetylphenyl)triazenes

M. D. Threadgill and M. F. G. Stevens

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Selective Reactions in the Triazene Series; I. Reduction of 1-(4-Acetylphenyl)-triazenes

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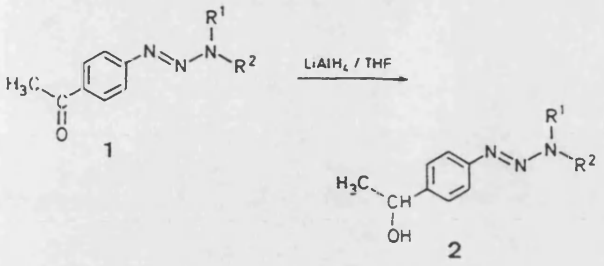
The 1-aryl-3,3-dimethyltriazenes have long been known to possess anti-tumour activity[1], but recent evidence points to their being prodrugs[2,3]. In the case of 1-(4-acetylphenyl)-3,3-dimethyltriazene (**1a**), H.P.L.C. analysis shows several major metabolites to be present in incubation mixtures of **1a** with mouse liver preparations[4,5]. It is likely that some metabolites could arise from biological reduction of the acetyl function to a 1-hydroxyethyl group. Authentic samples of the putative reduction products **2a–d** arising from **1a–d** were required and their synthesis has been achieved.

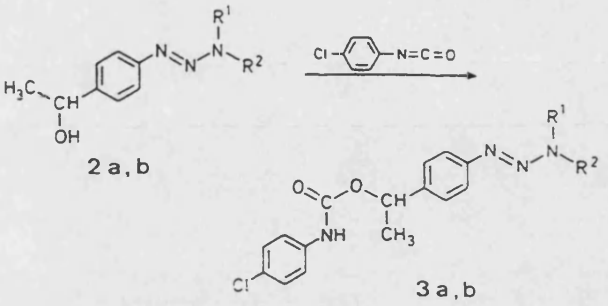
The traditional approach to the synthesis of aryl-triazenes, that of coupling an arenediazonium salt [here derived from 1-(4-aminophenyl)-ethanol] with a primary or secondary aliphatic amine, was considered to be inappropriate in this case since pentazadienes have been found when primary aliphatic amines are treated with arenediazonium salts bearing no other electron-withdrawing substituents[6].

A more biomimetic approach would involve selective reduction of the ketone moiety of the triazenes **1a–d**. Triazenes, being masked diazo-compounds, are very labile to metal/acid reducing conditions; indeed, a previous report of such treatment of a triazene showed reduction of the $-N{=}N-N\lessdot$ group while leaving a nitrile unscathed[7]. The problem encountered in the present work was therefore to achieve selec-

tive reduction of the the acetyl function of the triazenes **1a–d** without cleavage of the —N=N—N< link. Sodium borohydride was found to be without effect on acetylphenyltriazene **1a** but use of lithium aluminium hydride smoothly and rapidly gave the desired 1-hydroxyethyl derivatives **2a–d** in the cases of both di- and trisubstituted triazenes.



The substrates **1a–d** were prepared in the usual way by coupling 4-acetylbenzenediazonium chloride with the appropriate primary or secondary aliphatic amine. Derivatives of some of the triazenes were prepared to aid characterisation. In particular, the 3,3-dialkyl-triazenes **2a, b** were converted to their 4-chlorophenylaminocarbonyl derivatives **3a, b** by treatment with 4-chlorophenyl isocyanate in an aprotic solvent. The monoalkyl compounds **2c, d** were found to be very unstable to purification procedures and to attempted derivatisation; however, analysis of their ^{1}H-N.M.R. spectra indicated that they were obtained in a substantially pure state (>97%) from the work-up employed.



Resolution of the racemic compounds **2a–d, 3a, b** was not attempted since our biological interest lies in the effect of change in the electron-density in the triazene group rather than in geometry remote from this moiety.

These results show that a complex hydride agent offers a clean and efficient reduction of a substituted phenyltriazene while leaving the —N=N—N< functionality intact. The results of metabolic and anti-tumour experiments will be reported elsewhere; it would be expected that metabolically-generated 1-[4-(1-hydroxyethyl)-phenyl]-triazenes **(2a–d)** would be much less stable[8] than their precursor 1-(4-acetylphenyl)-triazenes **(1a–d)** and perhaps therefore be of different biological activity.

1-(4-Acetylphenyl)-3,3-dimethyltriazene (1a); Typical Procedure:
4′-Aminoacetophenone (2.02 g, 15 mmol) is suspended in water (12 ml) and 36% hydrochloric acid (7.5 ml) is added. The resulting slurry is

Table. Triazenes 1, 2, and 3 prepared

Product[a] No.	R^1	R^2	Yield [%]	m.p. [°C]	Molecular formula[b] or Lit. m.p. [°C]	^{1}H-N.M.R. ($CDCl_3$) δ [ppm]	M.S. m/e (M^+)
1a	CH_3	CH_3	68	92–93°	91–92°[9]	2.55 (s, 3H); 3.35 (br. s, 6H); 7.45 (d, 2H, J = 8 Hz); 7.90 (d, 2H, J = 8 Hz)	191
1b	C_2H_5	C_2H_5	98	oil	–	1.30 (t, 6H, J = 7 Hz); 2.55 (s, 3H); 3.80 (q, 4H, J = 7 Hz); 7.50 (d, 2H, J = 8 Hz); 7.95 (d, 2H, J = 8 Hz)	219
1b · semicarbazone			79	206–207°	$C_{13}H_{20}N_6O$ (276.3)	1.30 (t, 6H, J = 7 Hz); 2.25 (s, 3H); 3.80 (q, 4H, J = 7 Hz); 6.2 (br. s, 2H); 7.40 (d, 2H, J = 8 Hz); 7.70 (d, 2H, J = 8 Hz); 7.1 (br. s, 1H)[c]	276
1c	CH_3	H	78	89–90°	90–92°[6]	2.25 (s, 3H); 3.3 (br. s, 3H); 7.2–8.0 (m, 4H); 9.3 (br. s, 1H)	177
1d	C_2H_5	H	78	75–77° (dec.)	$C_{10}H_{13}N_3O$ (191.2)	1.30 (t, 3H, J = 7 Hz); 2.50 (s, 3H); 3.75 (q, 2H, J = 7 Hz); 7.2–8.0 (m, 4H); 9.2 (br. s, 1H)	191
2a[d]	CH_3	CH_3	~100	oil	$C_{10}H_{15}N_3O$ (193.2)	1.40 (d, 3H, J = 6 Hz); 2.7 (br. s, 1H); 3.30 (s, 6H); 4.80 (q, 1H, J = 6 Hz); 7.30 (s, 4H)	193
2b	C_2H_5	C_2H_5	93	oil	$C_{12}H_{19}N_3O$ (221.3)	1.25 (t, 6H, J = 7 Hz); 1.40 (d, 3H, J = 6 Hz); 2.8 (br. s, 1H); 3.70 (q, 4H, J = 7 Hz); 4.80 (q, 1H, J = 6 Hz); 7.35 (s, 4H)	221
2c	CH_3	H	91	oil	–	1.45 (d, 3H, J = 6 Hz); 3.2 (br. s, 1H); 3.25 (s, 3H); 4.85 (q, 1H, J = 6 Hz); 7.0 (br. s, 1H); 7.30 (s, 4H)	179
2d	C_2H_5	H	96	oil	–	1.25 (t, 3H, J = 7 Hz); 1.45 (d, 3H, J = 6 Hz); 3.0 (br. s, 1H); 3.65 (q, 2H, J = 7 Hz); 4.85 (q, 1H, J = 6 Hz); 7.30 (s, 4H); 8.0 (br. s, 1H)	193
3a	CH_3	CH_3	77	104–105°	$C_{17}H_{16}ClN_4O_2$ (346.8)	1.55 (d, 3H, J = 6 Hz); 3.35 (s, 6H); 5.90 (q, 1H, J = 6 Hz); 7.0 (br. s, 1H); 7.30 (s, 4H); 7.40 (s, 4H)	348, 346
3b[d]	C_2H_5	C_2H_5	73	oil	$C_{19}H_{23}ClN_4O_2$ (374.9)	1.20 (t, 6H, J = 7 Hz); 1.55 (d, 3H, J = 6 Hz); 3.75 (q, 4H, J = 7 Hz); 5.90 (q, 1H, J = 6 Hz); 7.0 (br. s, 1H); 7.25 (s, 4H); 7.40 (s, 4H)	376, 374

[a] All compounds were characterised by I.R., N.M.R., U.V., and M.S.
[b] Satisfactory microanalyses were obtained for new compounds where shown (C ±0.30, H ±0.26, N ±0.31).
[c] In DMSO-d_6.
[d] Purified by chromatography (alumina, diethyl ether).

cooled to 5 °C in an ice-bath and a solution of sodium nitrite (1.08 g, 15.6 mmol) in water (3 ml) is added at such a rate that the temperature does not rise above 10 °C. The resulting solution of the diazonium salt is poured during 5 min into 40% aqueous dimethylamine (30 ml) with vigorous stirring. The mixture is immediately extracted with chloroform (2 × 30 ml). The combined extracts are washed with water (4 × 30 ml), dried with sodium sulphate, filtered, and the solvent evaporated under reduced pressure. The residue is recrystallised from toluene/cyclohexane giving **1a** as long, pale buff needles; yield: 1.95 g (68%); m.p. 92–93 °C.

1-[4-(1-Hydroxyethyl)-phenyl]-3,3-dimethyltriazene (2a); Typical Procedure:

To a solution of 1-(4-acetylphenyl)-3,3-dimethyltriazene (**1a**; 1.91 g, 10 mmol) in tetrahydrofuran (30 ml) is added lithium aluminium hydride (380 mg, 100 mmol) during 5 min. The suspension is stirred for a further 10 min before addition of water (30 ml) (cautiously at first). The mixture is extracted with ethyl acetate (100 ml). This extract is washed with water (3 × 80 ml) and saturated brine (50 ml), dried with sodium sulphate, filtered and the solvent evaporated under reduced pressure to give **2a** as a colourless oil; yield: 1.93 g (100%).

$C_{10}H_{15}N_3O$	calc.	C 62.15	H 7.82	N 21.74
(193.2)	found	61.89	8.04	22.03

1-(4-Chlorophenylaminocarbonyloxy)-1-[4-(3,3-dimethyltriazen-1-yl)-phenyl]-ethane (3a); Typical Procedure:

A solution of 1-[4-(1-hydroxyethyl)-phenyl]-3,3-dimethyltriazene (**2a**; 386 mg, 2 mmol), 4-chlorophenyl isocyanate (307 mg, 2 mmol), and triethylamine (50 mg, 0.5 mmol) in tetrahydrofuran (5 ml) is boiled under reflux for 16 h. Evaporation of the solvent and of triethylamine under reduced pressure gives a buff solid which, on recrystallisation from aqueous methanol, furnishes **3a** as pale buff needles: yield: 520 mg (77%); m.p. 104–105 °C.

$C_{17}H_{19}ClN_4O_2$	calc.	C 58.88	H 5.52	N 16.15
(346.8)	found	58.75	5.55	15.84

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PUBLICATION 8

(±)-2,3-Dihydroxypropyl Dichloroacetate, an Inhibitor of Glycerol Kinase

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(±)2,3,-DIHYDROXYPROPYL DICHLOROACETATE, AN INHIBITOR OF GLYCEROL KINASE

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Of a range of glycerol analogues, (±)-2,3-dihydroxypropyl dichloroacetate (III) has been shown to be the most potent inhibitor of glycerol kinase *in vitro*. Inhibition is noncompetitive with a Ki value of 1.8×10^{-3} *M*. The presence of ATP seems essential for effective inhibition of the enzyme, suggesting that the inhibitor is phosphorylated to a glycerol-3-phosphate analogue. *In vivo* III causes a decrease in the specific activity of liver glycerol kinase and produces a dose-dependent reduction in blood glucose levels. There is a reduction in the conversion of [U-^{14}C] glycerol into glucose after administration of III to CBA/CA mice while gluconeogenesis from fructose is increased. This suggests that of the enzymes of gluconeogenesis only glycerol kinase is inhibited by III. This compound may be useful in reducing the lipid contribution to gluconeogenesis in advancing cancer.

INTRODUCTION

Many tumors have an absolute requirement for glucose as an energy supply, either because of enzymatic differences (Weinhouse, 1973), or through a low oxygen tension(Thomlinson and Gray, 1955) which would prevent oxidation of nutrients by molecular oxygen and lead to a dependence on glycolysis to supply the energy demands. Thus a tumor behaves as a trap for glucose and acts as a powerful hypoglycemic factor imposing a strain on the host's ability to maintain normal blood glucose levels (Shapot, 1972). Despite this tendency towards hypoglycemia normal blood glucose concentrations have been observed in most tumor-bearing animals and patients. This indicates an increased dependence on gluconeogenesis from non-carbohydrate precursors by the host (Gold, 1974). An increased Cori cycle activity is present in patients with progressive malignant disease and weight loss (Holroyde *et al.*, 1975; Waterhouse, 1974) which could convert the large amounts of lactate produced by tumors back into glucose (Gold, 1966; Shapot and Blinov, 1974). There is also an enhanced activity of gluconeogenic enzymes in the liver of tumor-bearing animals (Gutman, Thilo and Biran, 1969). Since the rate of gluconeogenesis in tumors is low (Morris, 1975), all resynthesis of glucose must occur in host tissues, particularly liver and kidney.

Gold (1974) has proposed that inhibitors of gluconeogenesis may be tumor growth inhibitors. Hydrazine sulfate, an inhibitor of gluconeogenesis at the phosphoenolpyruvate carboxykinase reaction, shows antitumor activity (Gold, 1975). The antitumor effect of hydrazine sulfate is potentiated by clofibrate, a substance which lowers blood triglycerides (Gold, 1978). This combination would be expected to inhibit gluconeogenesis from both lactate and amino acids as well as reducing the supply of glycerol for conversion to glucose. It has been suggested that there is a substantial lipid contribution to gluconeogenesis in advancing cancer (Begg, 1955). The glycerol concentration in the plasma from cancer patients has been shown to be

three times higher than that from control subjects and the glycerol turnover rate has also been shown to be significantly higher in cancer patients (Lundholm *et al.*, 1982). However, there is no useful agent which interferes with the conversion of glycerol to glucose.

Glycerol passes into the gluconeogenic pathway via phosphorylation to α-glycerol-L phosphate mediated by glycerol kinase (ATP): glycerol phosphotransferase, EC 2.7.1.30) followed by oxidation to dihydroxyacetone phosphate by the NAD-dependent glycerophosphate dehydrogenase (syn glycerol-3-phosphate: NAD 2-oxidoreductase, EC 1.1.1.8). This study reports on the effect of derivatives of glycerol on glycerol kinase activity *in vitro* as well as the effect on glycerol conversion to glucose *in vivo*.

MATERIALS AND METHODS

[U-^{14}C]Glycerol (sp. act. 171 mCi/mmol) and D-[U-^{14}C]fructose (sp. act. 330 mCi/mmol) were obtained from Amersham International PLC., Bucks. *E. coli* glycerol kinase was purchased from Sigma Chemical Co., Dorset.

(±)-2,3-Dihydroxypropyl Chloroacetate

Propan-1,2,3-triol (18.4 g), chloroacetic acid (18.9 g) and 88% phosphoric acid (1.0 ml) were suspended in toluene (100 ml). This mixture was boiled under reflux with a Dean and Stark water separator until water evolution ceased (3 h) before being allowed to stand at ambient temperature for 16 h. Sodium hydrogen carbonate (24 g) was added, followed by methanol (300 ml). The suspension was filtered and the solvents were removed from the filtrate by evaporation under reduced pressure. The residue was distilled at 4 torr and the fraction boiling at 195–200°C was collected as 2,3-dihydroxypropyl chloroacetate [lit (Merry, 1981) $BPt_{0.1}$ 130°C]. Yield 24.1 g (71.5%). γ_{max} (liq. film): 3300 (br), 1745 cm^{-1}. δ[$(CD_3)_2SO+D_2O$]: 3.6–3.8 (3H) m $C(3)H_2$+C(2)H; 4.15(2H) d (J=7 Hz) $C(1)H_2$; 4.3 (2H)s $OCOCH_2Cl$.

(±)-2,3-Dihydroxypropyl Dichloroacetate

Prepared as for (±)-2,3-dihydroxypropyl dichloroacetate using propan-1,2,3-triol (18.4 g), dichloroacetic acid (25.8 g), 88% phosphoric acid (1.0 ml) and toluene (150 ml). Fraction BPt_3 220-8°C collected [lit (Merry, 1981) $BPt_{0.2}$ 159°C]. Yield 31.2 g (77%). γ_{max} (liq. film): 3350 (br), 1760 cm^{-1}. δ[$(CD_3)_2SO+D_2O$]: 3.6–3.8 (3H)m $C(3)H_2$+C(2)H; 4.35 (2H) d (J=7 Hz) $C(1)H_2$; 6.2 (1H)s $OCOCHCl_2$.

(±)-1-Chloropropan-2,3-diol was purchased from Aldrich Chemical Co. Ltd., and (±)-2,3-dihydroxypropyl acetate from Koch-Light Ltd.

Inhibition of glycerol kinase activity The incubation mixture consisted of 50 mM Tris-HCl, pH 7.0, 2 mM $MgCl_2$, 0.5 μmole ATP, glycerol kinase (0.24 U/assay) plus or minus the inhibitor. After incubation at 37°C for 10 min the reaction was initiated with 25 nmoles of [U^{14}C]glycerol (sp. act. 0.01 m Ci/mmol) to give a final reaction volume of 0.5 ml. After a further 10 min incubation the reaction was terminated with 0.5 ml of cold 2 M glycerol, followed by thorough mixing. Samples (50 μl) were spotted on DE 81 phosphocellulose discs (Whatman, Maidstone, Kent) and the discs were washed extensively in 0.1 M glycerol. Essentially all of the [^{14}C]glycerol-3-phosphate was retained by the filters, while the excess radioactive glycerol was

removed. The discs were eluted with 1 ml of 0.1 N HCl in scintillation vials and the radioactivity determined in PCS scintillation fluid (Hopkin and Williams).

Animal studies *In vivo* experiments with (±)-2,3-dihydroxypropyl dichloroacetate (III) were performed on CBA/CA mice which were starved for 24 h prior to the experiments. (±)-2,3-dihydroxypropyl dichloroacetate was dissolved in 10% DMSO in arachis oil and administered by i.p. injection. Controls received oil alone. For the determination of liver glycerol kinase, livers were removed from mice 2 h after injection of drug and homogenized in 3 ml of 150 m*M* Tris-HCl, pH 7.5, containing 5 m*M* $MgCl_2$, 25 m*M* 2-mercaptoethanol and 1 m*M* EDTA. The supernatant fluid, obtained after centrifugation at 18,000×*g* for 30 min, was assayed for glycerol kinase as described above. For the determination of glucose blood samples were taken from the tail vein at 1 h intervals, and the level of glucose was measured using dextrostix.

Separation of glucose from fructose and glycerol in blood filtrates Formation of glucose from precursors was determined essentially by a method of Friedmann, Goodman and Weinhouse (1970). Mice were injected with [U-^{14}C] glycerol (10 mg, 2.5 μCi/animal) or D-[U-^{14}C]fructose (10 mg, 3.2 μCi/animal) 2 h after drug injection. Blood samples were taken by heart puncture 30 min after injection of label. A protein-free filtrate was prepared from 1 ml blood, 1.5 ml water, 1.5 ml 0.3*M* $Ba(OH)_2$ and 3 ml 5% $ZnSO_4$. After centrifugation the supernatant was passed through a 9×0.6 cm column of Amberlite MB-3 HCO_3^- and the column was washed with water to a volume of 10 ml. The filtrate was allowed to stand overnight with 1 mg of carrier glucose and 2 mg (20 U) of glucose oxidase and the gluconic acid formed was neutralized to a phenolphthalein end-point with 1 m*M* NaOH. The entire solution was passed over a 6×0.6 cm column of Amberlite CG-400, 100–200 mesh and the column was washed to an eluate volume of 15 ml. Gluconic acid was removed from the resin by washing with 15 ml 0.1*M* NaCl. Radioactivity in the two fractions was determined in PCS scintillation fluid.

RESULTS

(±)-2,3-Dihydroxypropyl acetate (I), (±)-2,3-dihydroxypropyl chloroacetate (II) and (±)-2,3-dihydroxypropyl dichloroacetate (III) were effective inhibitors of glycerol kinase *in vitro*, the respective ED_{50} (concentration required for 50% inhibition of enzyme activity) being 1.32, 0.88 and 0.33 m*M* respectively. There was no inhibition by 1-chloro-2,3-propane diol or 2,2,4-trimethyl-4-(phenyloxymethyl)dihydrodioxole at concentrations up to 25 m*M*. Maximum inhibition was seen with ATP in the inhibition assay; the ED_{50} for III was increased to 1.11 m*M* in the absence of ATP. This suggests the possibility of phosphorylation of the glycerol analogues, followed by product inhibition of the enzyme, and explains the need for a free terminal primary alcohol group. Lineweaver-Burk plots (Figure 1) indicated that III inhibited glycerol kinase noncompetitively with an apparent inhibition constant, K_i, of $1.8\times10^{-3}M$. The Km of the purified enzyme towards glycerol was 0.4 m*M*. In the absence of glycerol inhibition of glycerol kinase by III increased with time of incubation.

Formation of α-glycerophosphate by mouse liver glycerol kinase increased linearly with reaction time and was proportional to the protein concentration in the assay (Figure 2). Pretreatment of mice for 2 h with III caused a decrease in the specific

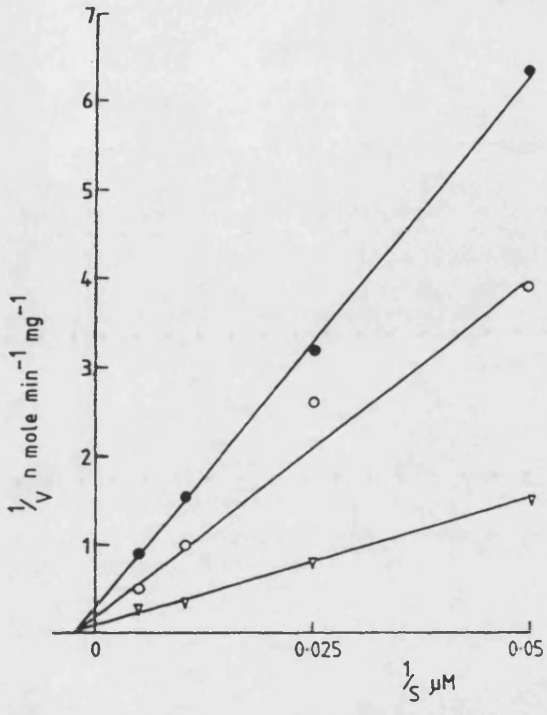

FIGURE 1 Double reciprocal plot of the initial velocity of glycerol kinase versus glycerol concentration in the absence (∇—∇) and in the presence of 1.6 (○—○) and 2 mM (○—○) glycerol diacetylchloride.

activity of the enzyme which increased with increasing concentrations of the inhibitor (Figure 2). These results suggest that III could be an effective inhibitor of gluconeogenesis from glycerol *in vivo*.

The effect of III on precursors for gluconeogenesis is shown in Table I. Increasing concentrations of III caused a reduction in the ratio of glucose to glycerol indicating inhibition of gluconeogenesis from glycerol, while the glucose/fructose ratio increased, except at the highest dose employed. At 625 mg/kg the glucose/fructose ratio was significantly elevated while the glucose/glycerol ratio was unaltered. These results suggest that of the enzymes of gluconeogenesis only glycerol kinase is inhibited by III.

TABLE I
Effect of (±)-2,3-dihydroxypropyl dichloroacetate on gluconeogenesis from [U-^{14}C] glycerol and D-[U-^{14}C] fructose in CBA/CA mice

Dose mg/kg	^{14}C glucose/^{14}C glycerol	^{14}C glucose/^{14}C fructose
0	5.3±0.3	3.73±0.6
313	5.3±0.5	3.33±0.5
625	5.5±0.7	6.1 ±0.7
1250	0.4±0.05	5.3 ±0.5
2500	0.4±0.1	1.2 ±0.1

The results in Figure 3 depict the hypoglycemic effect obtained with III in mice which had been starved for 24 h after i.p. injections of 250,500, 1000, 2500 or 5000 mg/kg. A significant decrease in blood glucose between control and treated groups was obtained 3 h after treatment. With the two higher doses (1000 and 2500 mg/kg) the effect persisted over a 5 h period, while with 5000 mg/kg the mice died. Hence the magnitude and duration of the hypoglycemic effect are dose-dependent.

There was no effect of III on $^{14}CO_2$ release from D-(U-^{14}C) glucose by mouse TLX5 lymphoma cells *in vitro* at concentrations below 20 mg/ml.

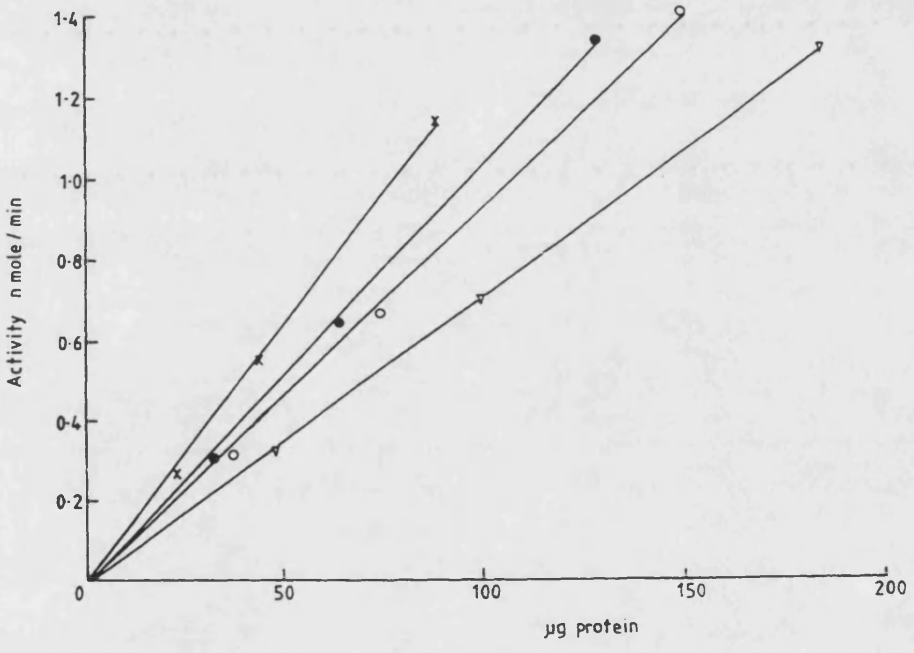

FIGURE 2 Effect of (±)-2,3-dihydroxypropyl dichloroacetate on mouse liver glycerol kinase. Mice were injected i.p. with 625 (•—•), 1250 (○—○) or 2500 mg/kg (▽—▽) of (±)-2,3-dihydroxypropyl dichloroacetate or vehicle alone (x—x) and the activity of liver glycerokinase was assayed 2 h afterwards.

DISCUSSION

Gluconeogenesis from glycerol may be of importance in cancer patients demonstrating hyperlipemia or in subjects fed a high lipid diet to induce ketosis (Magee *et al.*, 1979). We have been attempting to reduce the supply of glucose to the tumor either by dietary or other means and for this reason analogues of glycerol have been investigated as potential inhibitors of glycerol kinase, the first enzyme in the pathway from glycerol to glucose. One such analogue, (±)-2,3-dihydroxypropyl dichloroacetate (III), proved to be an effective inhibitor of this enzyme, both *in vitro* and *in vivo*. Blood glucose levels are rapidly reduced after administration of this agent. The decrease in blood glucose probably arises from an inhibition of gluconeogenesis from glycerol, since gluconeogenesis from D-fructose is increased under such conditions and glycolysis is not affected. At 625 mg/kg of III gluconeogenesis from fructose is stimulated without a significant reduction of gluconeogenesis from glycerol. High

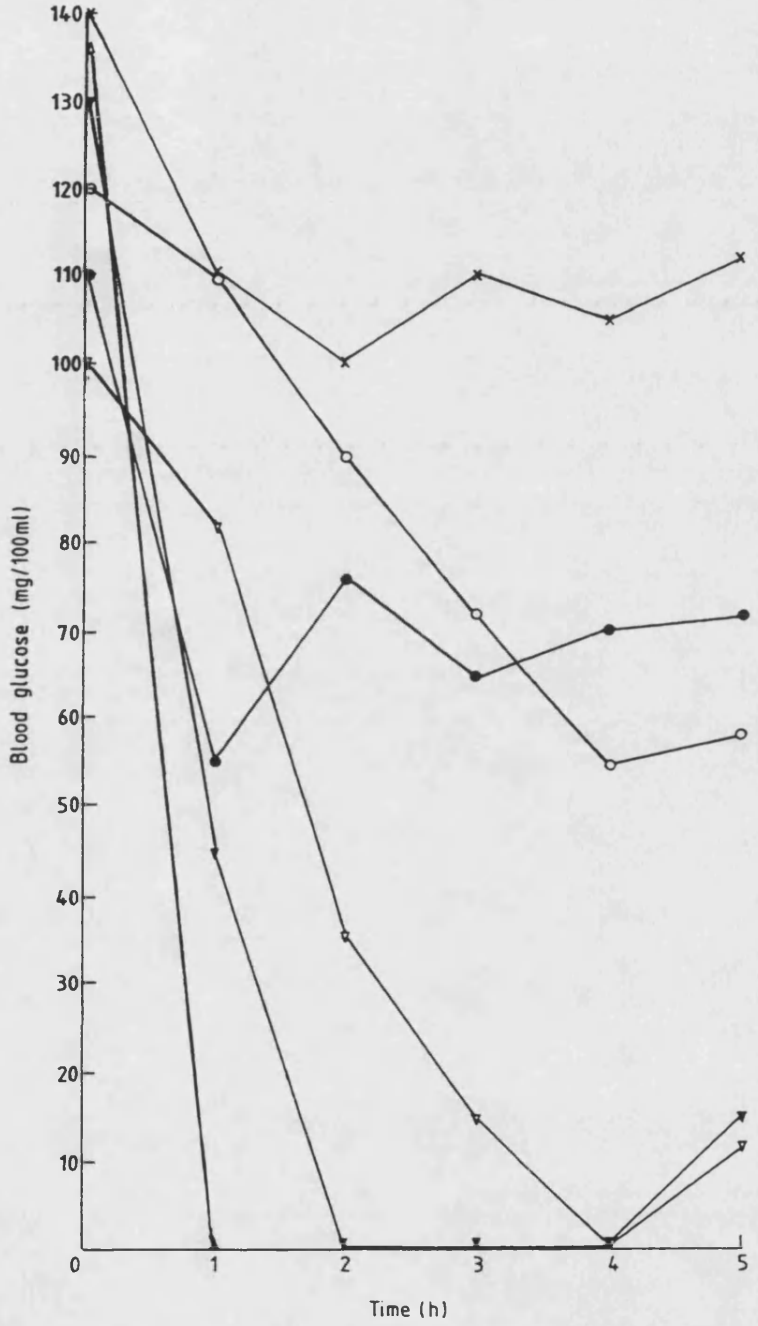

FIGURE 3 Effect of (±)-2,3-dihydroxypropyl dichloroacetate on blood glucose levels. Mice were injected either with vehicle alone (x—x) or with 250 (•—•), 500 (○—○) 1000 (▽—▽), 2500 (▼—▼) or 5000 (△—△) mg/kg of (±)-2,3-dihydroxypropyl dichloroacetate. Samples (50 µl) of blood were taken from the tail vein at 1 h intervals and the concentration of glucose was determined using dextrostix. Data for each dose studied were taken from a single animal. The experiment was repeated five times and the results are the data for a typical experiment.

doses of III cause a rapid decrease in blood glucose and death within 2 h. The toxicity of III is probably not due to inhibition of the glycolytic pathway since pharmacological concentrations had no effect on $^{14}CO_2$ release from D-[U-^{14}C] glucose by TLX5 mouse lymphoma cells *in vitro*. It is possible, however, that III could become incorporated into membrane lipids.

Recent results (Graziani, Erikson and Erikson, 1983) provide evidence that pp60$^{v\text{-}src}$, the Rous sarcoma virus transforming gene product, is associated with glycerol kinase activity. Although the physiological significance of this enzyme production has still to be elucidated, it seems probable that inhibitors of glycerol kinase activity may directly subvert the transformation associated processes.

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PUBLICATION 9

The Formation and Metabolism of *N*-Hydroxymethyl Compounds. Part 6. The Synthesis of *S*-(Amidomethyl)-, *S*-(Ureidomethyl)- and *S*-(1,3,5-Triazin-2-ylmethyl)-Glutathione derivatives

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The Formation and Metabolism of *N*-Hydroxymethyl Compounds. Part 6.[1] The Synthesis of *S*-Amidomethyl-, *S*-Ureidomethyl-, and *S*-(1,3,5-Triazin-2-ylaminomethyl)-glutathione Derivatives

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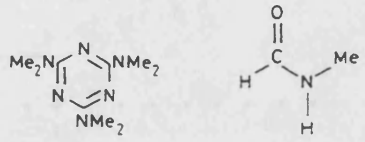
Treatment of *N*-hydroxymethyl and *N*-alkoxymethyl compounds with glutathione or *N*-acetylcysteine in trifluoroacetic acid affords the corresponding glutathione or *N*-acetylcysteine derivatives in high yield. Alkoxymethylureas are formed by the condensation of ureas with formaldehyde and alcohols under basic conditions; the implications of this observation are discussed with reference to possible biochemical mechanisms.

N-Hydroxymethyl compounds are produced by the metabolism of drugs and other materials containing *N*-methyl groups, by preparations of murine liver.[2] It has been reported that, for example, the antitumour agents hexamethylmelamine (**1**)[3] and *N*-methylformamide (**2**)[4] and the herbicide Monuron [*N*-(4-chlorophenyl)-*N'*,*N'*-dimethylurea] (**3**)[5] are hydroxylated by this route which involves cytochrome P450. These hepatic metabolites containing the carbinolamine moiety may act as electrophiles, either through the intermediacy of a small equilibrium concentration of the corresponding iminium ion (a dehydration product) or through biological derivatisation of the alcohol (*e.g.* sulphation, acetylation) which increases its leaving group ability. As electrophiles, they may be predicted to be conjugated *in vivo* (either enzymatically or chemically) to glutathione (γ-glutamylcysteinylglycine)[6] and hence be excreted as the glutathione conjugate (**4**) itself or as the corresponding mercapturic acid (**5**). Any or all of these compounds may be electrophiles in their own right (with RS^- as a leaving group), or may act as transport forms of formaldehyde and thus be present as toxic or carcinogenic metabolites. We sought therefore to prepare a range of such *S*-aminomethyl compounds.



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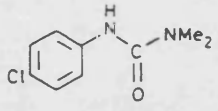
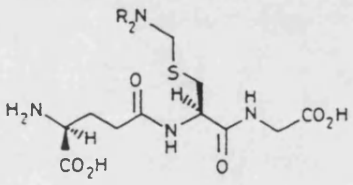
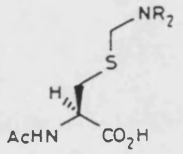


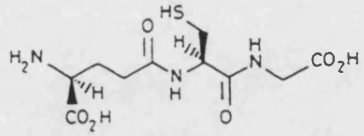
(3)



(4)



(5)

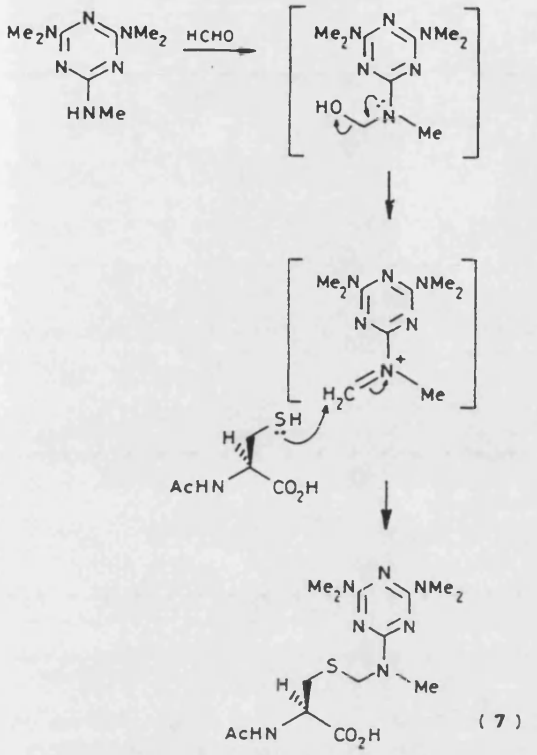


(6)

The synthesis of one such *S*-aminomethylglutathione derivative has been reported recently,[7] albeit in low yield, from the Mannich-type condensation of 4-aminoazobenzene with formaldehyde and glutathione (**6**) in an aqueous medium. A modification of this technique enabled us to prepare the *N*-acetylcysteine–melamine adduct (**7**) in good yield. Glutathione is insoluble in the aqueous methanol employed and, not surprisingly, failed to react under these conditions. The mechanism presumably involves a methylene-iminium ion (Scheme 1). It is interesting to note that benzamide did not condense with *N*-acetylcysteine and formaldehyde under these conditions, the starting materials being recovered; thus any *N*-hydroxymethylbenzamide formed is not in equilibrium with sufficient imine or iminium species to effect condensation.

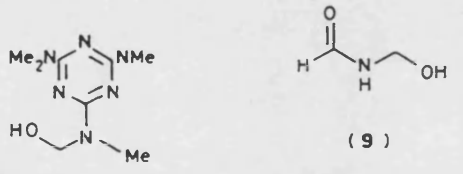
However, dissolution of equimolar amounts of preformed *N*-hydroxymethyl-*N*,*N'*,*N'*,*N''*,*N''*-pentamethylmelamine (**8**) or *N*-hydroxymethylamides (**9**) and (**10**) in *ca.* 0.7M-solutions of glutathione in anhydrous trifluoroacetic acid (TFA), followed immediately by evaporation of the solvent under reduced pressure, gave the condensation products in consistently high yields. The use of TFA is apposite in that it acts both as a good solvent for the otherwise troublesome glutathione and as an acid catalyst of low nucleophilicity for the generation of iminium ions for capture by the nucleophilic thiol. The condensation of *N*-acetylcysteine with *N*-hydroxymethyl-4-t-butylbenzamide was similarly effected. It proved impossible to generate the methylene-iminium ion under these conditions by elimination of water in the reverse sense from 4-chloro-*N*-methylbenzohydroxamic acid[8] (**12**) as shown in Scheme 2.

The *N*-hydroxymethylamides (**9**) and (**10**) and *N*-hydroxymethyl(pentamethyl)melamine (8 g) used above were prepared without difficulty in the usual way from the corresponding NH compound, formaldehyde, and base in an appropriate solvent. However, on attempting to prepare the *N*-hydroxymethylureas (**11**), we were unable to repeat the work of Zigeuner *et al.*[9] who warmed phenylurea (**13a**) with paraformaldehyde and sodium



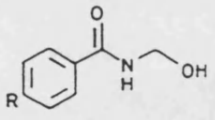
(7)

Scheme 1. Proposed mechanism of formation of compound (7) *via* the methylene-iminium ion



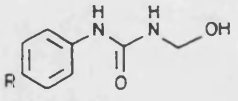
(8)

(9)



(10)

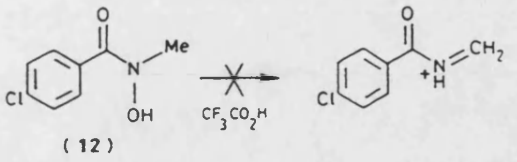
a; R = Bu^t
b; R = H



(11)

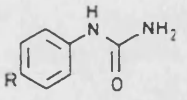
a; R = H
b; R = Cl

hydroxide in methanol. In our hands, the sole isolable product was *N*-methoxymethyl-*N'*-phenylurea in very low yield. Increasing the reaction time and temperature enabled the three representative alkoxymethylureas (**14a—c**) to be synthesised smoothly in the appropriate alcohols, as in Scheme 3. Alkoxymethylureas are reported[9] to be formed when *N*-hydroxymethylureas are treated with an alcoholic hydrogen chloride solution, thus favouring the necessary iminium ion formation; nevertheless, a small but significant equilibrium concentration of arylurea methylene-iminium or methylene-



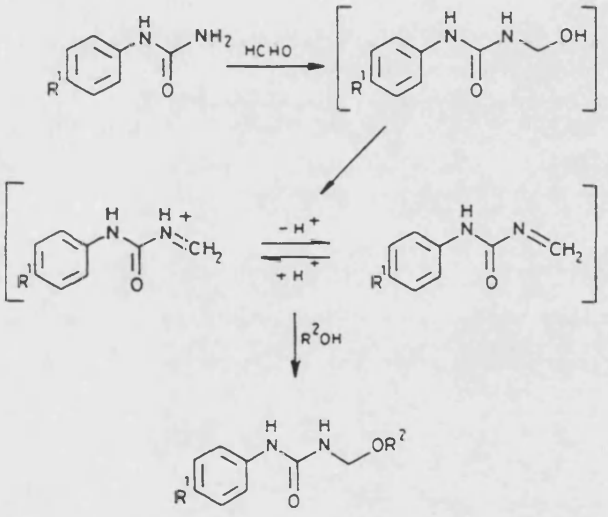
(12)

Scheme 2. [4-Chloro-*N*-methylbenzohydroxamic acid (**12**) is unreactive towards nucleophiles in CF_3CO_2H.]



(13)

a; R = H
b; R = Cl
c; R = NO_2



(14)

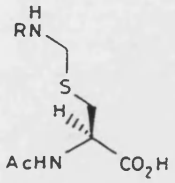
a; R^1 = H, R^2 = Et
b; R^1 = Cl, R^2 = Me
c; R^1 = NO_2, R^2 = Me

Scheme 3. Formation of the alkoxymethylureas (**14a—c**)

imine moieties must be present even under the mildly basic conditions of our experiments.

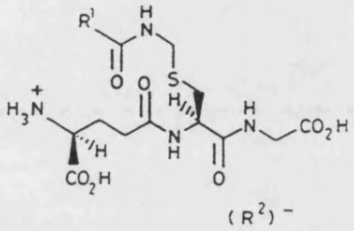
The alkoxymethylureas (**14a**) and (**14b**) are found to react with glutathione, and (**14a**) with *N*-acetylcysteine, as readily as the *N*-hydroxymethylamides. Direct ^1H n.m.r. monitoring of these reaction mixtures reveals that the condensation is complete within 2 min, but that only after 20 min has all the methanol or ethanol released been esterified by the trifluoroacetic acid; this implies that the rate of the reversible methylene-iminium ion formation is unaffected by the rate at which the alcohol is irreversibly sequestered.

The structural assignment of the synthetic conjugates is based on spectroscopic data. The characteristic feature of the ^1H n.m.r. spectrum of compounds (**15**) and (**16**) [$(CD_3)_2SO$; 220 MHz] is the resonance of the NCH_2S moiety which appears as two separate sets of signals, indicating that the prochiral



(15)

a; R = 4-Bu^{t}C$_6$H$_4$CO
b; R = PhNHCO



(16)

a; R^1 = H, R^2 = $CF_3CO_2^-$
b; R^1 = 4-Bu^{t}C$_6$H$_4$, R^2 = picrate$^-$
c; R^1 = Ph, R^2 = $CF_3CO_2^-$
d; R^1 = PhNH, R^2 = $CF_3CO_2^-$
e; R^1 = 4-ClC$_6$H$_4$NH, R^2 = $CF_3CO_2^-$

methylene group is in an asymmetric environment. This effect is well illustrated in the ^{1}H n.m.r. spectrum of compound (**16b**) in which the NCH_2S resonances appear at δ 4.46 (1 H, dd, *J* 13 and 6.5 Hz) and 4.57 (1 H, dd, *J* 13 and 6.5 Hz). Treatment with deuterium oxide removes the corresponding NH triplet at δ 9.16 and its 6.5 Hz coupling, leaving the 13 Hz geminal coupling typical of an asymmetric methylene group; this contrasts with the corresponding 2 H singlet in the spectrum of the achiral substrate (**10a**). As expected, the cysteine β-CH_2 is prochiral. The coupling constants in the spectrum of the benzamidomethyl glutathione compound (**16c**) are typical, with geminal coupling constants $J_{\beta1,\beta2}$ 14 Hz, $J_{\beta1,\alpha}$ 10.5 Hz, and $J_{\beta2,\alpha}$ 4 Hz. From a simplified Karplus analysis, it can be deduced that the molecule adopts one of the staggered conformations about the C_α–C_β bond shown in the Figure. As predicted from steric considerations, the benzamidomethylthio group is *gauche* to one of the peptide links and *trans* to the other (Figure). The ^{1}H n.m.r. spectrum of the formamide (**16a**) was more complex, indicating approximately equal populations of two rotamers about the formamide carbonyl–nitrogen bond.

Two main conclusions can be drawn from these results. Firstly, since alkoxymethylureas are formed under basic conditions and are stable under such conditions, there must be a small but significant equilibrium proportion of the corresponding iminium ions or imines under these very basic conditions. It is therefore reasonable to postulate that an equal or higher equilibrium proportion of iminium ions or imines is present under the much less basic physiological conditions (pH 7.4). Hence it is feasible that the methylene-iminium ions formed directly from the dehydration of *N*-(4-chlorophenyl)-*N'*-hydroxymethylurea, a known metabolite of Monuron,[5] or from *N*-hydroxymethyl(pentamethyl)melamine may, as proposed[10] for *N*-hydroxymethylamines (in which iminium ion formation is more favoured), be the actual electrophile responsible for biological activity (mutagenic,[11] antineoplastic, or antibacterial[10] respectively). Secondly, it is shown here that a rapid, facile

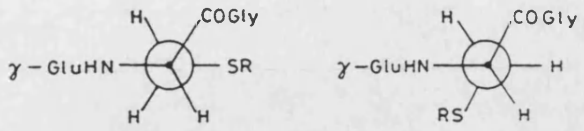


Figure. Newman projections of the C_α–C_β bond of the *S*-benzamidomethylglutathione (**16c**) conformers established by ^{1}H n.m.r. spectroscopy (R = $PhCONHCH_2$)

synthesis of glutathione conjugates, putative metabolites of some xenobiotic *N*-methyl compounds, is available. Since conjugation to glutathione is a common fate of hepatically generated electrophiles, it is important to have such authentic material for chemical and biochemical study. No attempt has been made to prepare the free glutathione forms of the glutathione derivatives from the salts, since the former would be expected to be released upon dissolution of the salts in the buffered aqueous media required for biochemical experiments.

Experimental

I.r. spectra were determined as Nujol mulls, except where otherwise stated. ^{1}H N.m.r. spectra were obtained at 60 MHz using a Varian EM360A spectrometer and at 220 MHz using a Perkin-Elmer R34 instrument and ^{13}C n.m.r. spectra were obtained with a Bruker WH-180, using tetramethylsilane as internal standard. M.p.s are uncorrected.

N-(4-*Chlorophenyl*)*urea* (**13b**).—This compound was prepared in 81% yield according to the general method of Furniss *et al.*[12] and had m.p. 208—210 °C (lit.,[13] 204—206 °C).

N-(4-*Nitrophenyl*)*urea* (**13c**).—4-Nitrobenzoic acid (8.35 g, 50 mmol) and phosphorus pentachloride (10.4 g, 50 mmol) were heated together at 120 °C until gas evolution ceased. Toluene (15 ml) was added and the mixture was heated to 205 °C during which process all volatile materials were distilled off (mainly toluene and phosphorus oxychloride). On being cooled, the crystalline residue was dissolved in acetone (200 ml) and was added to sodium azide (10.0 g, 154 mmol) and sodium hydrogen carbonate (1.0 g) in water (40 ml). This mixture was stirred for 2 h before being extracted with dichloromethane (2 × 200 ml). The combined organic extracts were dried (Na_2SO_4), filtered, and the solvents evaporated under reduced pressure to give almost pure 4-nitrobenzoyl azide as pale yellow prisms ($\nu_{max.}$ 2 180, 2 120, and 1 675 cm^{-1}). This azide, in toluene (60 ml), was boiled under reflux for 10 min after which a small evaporated sample showed $\nu_{max.}$ 2 250 cm^{-1}, corresponding to 4-nitrophenyl isocyanate. The cooled toluene solution was added to a large excess of ethereal ammonia giving an immediate yellow precipitate. Recrystallisation from aqueous methanol yielded the urea (**13c**) (3.30 g, 37%) as lemon yellow needles, m.p. 214—215 °C (lit.,[14] 215 °C) δ_H [60 MHz; $CDCl_3$–$(CD_3)_2SO$; 1:3] 6.1 (2 H, br, NH_2), 7.65 (2 H, d, *J* 9 Hz, ArH), 8.12 (2 H, d, *J* 9 Hz, ArH), and 9.2 (1 H, br, NH).

N'-Ethoxymethyl-N-phenylurea (**14a**).—Paraformaldehyde (2 g, 66.7 mmol of HCHO) was added to phenylurea (2.72 g, 20 mmol) and sodium hydroxide (100 mg, 2.5 mmol) in a mixture of ethanol (50 ml) and water (1 ml). The resulting suspension was boiled under reflux for 1.5 h before evaporation of the solvent under reduced pressure. Recrystallisation of the residue from aqueous ethanol furnished the *ethoxymethylurea* (**14a**) (3.41 g, 84%) as white needles, m.p. 105—107 °C (Found: C, 61.7; H, 7.1; N, 14.6. $C_{10}H_{14}N_2O_2$ requires C, 61.85; H, 7.25; N, 14.4%); $\nu_{max.}$ 3 250 and 1 660 cm^{-1}; δ_H (60 MHz; $CDCl_3$) 1.12 (3

H, t, *J* 7 Hz, CH_2CH_3), 3.48 (2 H, q, *J* 7 Hz, OCH_2CH_3), 4.60 (2 H, d, *J* 7 Hz, NCH_2O), 6.86 (1 H, t, *J* 7 Hz, $CONHCH_2$), 7.3 (5 H, m, ArH), and 8.1 (1 H, s, ArNH).

N-(4-*Chlorophenyl*)-N'-*methoxymethylurea* (**14b**).—Paraformaldehyde (3.0 g, 100 mmol of HCHO) and aqueous sodium hydroxide (10% w/v; 1.5 ml, 3.75 mmol) were added to *N*-(4-chlorophenyl)urea (**15b**) (5.12 g, 30 mmol) in methanol (60 ml). This suspension was boiled under reflux for 5 h before being cooled to 0 °C for 16 h. The solids were isolated by filtration and washed with a small volume of cold methanol to give the *methoxymethylurea* (**14b**) as white needles (5.08 g, 78%), m.p. 126—128 °C (Found: C, 50.35; H, 5.3; N, 12.8. $C_9H_{11}ClN_2O_2$ requires C, 50.35; H, 5.15; N, 13.05%); $\nu_{max.}$ 3 400, 3 300, and 1 630 cm^{-1}; δ_H (60 MHz; $CDCl_3$) 3.30 (3 H, s, OMe), 4.60 (2 H, d, *J* 7 Hz, NCH_2O), 6.93 (1 H, t, *J* 7 Hz, $NHCH_2O$), 7.22 (2 H, d, *J* 9 Hz, ArH), 7.45 (2 H, d, *J* 9 Hz, ArH), and 8.7 (1 H, br, ArNH).

N-(4-*Nitrophenyl*)-N'-*methoxymethylurea* (**14c**).—Aqueous formaldehyde solution (37% w/v; 6.0 ml, 74 mmol) and paraformaldehyde (2.7 g, 90 mmol of HCHO) were added to *N*-(4-nitrophenyl)urea (**13c**) (530 mg, 2.9 mmol) and potassium hydroxide (640 mg, 11.4 mmol) in methanol (50 ml). This suspension was boiled under reflux for 3 h before evaporation of the solvent under reduced pressure. Recrystallisation of the residue from methanol afforded the *methoxymethylurea* (**14c**) (310 mg, 44%) as pale yellow needles, m.p. 163.5 °C (decomp.) (Found: C, 48.1; H, 5.1; N, 18.5. $C_9H_{11}N_3O_4$ requires C, 48.0; H, 4.9; N, 18.65%); $\nu_{max.}$ 3 250 and 1 675 cm^{-1}; δ_H [60 MHz; $CDCl_3$–$(CD_3)_2SO$, 20:1] 3.37 (3 H, s, OMe), 4.70 (2 H, d, *J* 7 Hz, NCH_2O), 6.9 (1 H, br, NH), 7.70 (2 H, d, *J* 9 Hz, ArH), 8.20 (2 H, d, *J* 9 Hz, ArH), and 9.1 (1 H, br, NH).

N-*Acetyl*-S-(4-*t-butylbenzamidomethyl*)*cysteine* (**15a**).—*N*-Acetyl-L-cysteine (326 mg, 2 mmol) in trifluoroacetic acid (3 ml) was added to *N*-hydroxymethyl-4-t-butylbenzamide (**10a**)[1] (414 mg, 2 mmol). This mixture was stirred for 5 min at 20 °C before evaporation of the solvent at 35 °C and 1 Torr. The residue, in dichloromethane (20 ml), was washed with water (10 ml). The solution was dried (Na_2SO_4), filtered and the solvent evaporated to give a colourless gum. Column chromatography (silica gel; $CHCl_3$ with MeOH increasing from 0 to 15%) gave 4-t-butylbenzamide (90 mg, 26%) as a white powder identical with an authentic sample.[15] Evaporation of the solvents from later eluates afforded the *cysteine derivative* (**15a**) (413 mg, 59%) as a white powder which decomposed on gentle heating (Found: C, 57.6; H, 6.7; N, 7.7. $C_{17}H_{24}N_2O_4S$ requires C, 57.95; H, 6.85; N, 7.95%); $\nu_{max.}$ 3 300, 3 100, 1 715, and 1 665 cm^{-1}; δ_H [220 MHz; $(CD_3)_2SO$] 1.30 (9 H, s, CMe_3), 1.87 (3 H, s, Ac), 2.95 (1 H, dd, *J* 7 and 13 Hz), and 3.16 (1 H, dd, *J* 4 and 13 Hz) (cysteine CH_2), 3.5 (1 H, br, CO_2H), 4.35 (1 H, dt, *J* and 7 Hz, cysteine α-H), 4.48 (1 H, dd, *J* 6 and 13 Hz) and 4.52 (1 H, dd, *J* 6 and 13 Hz) (NCH_2S), 7.56 (2 H, d, *J* 8 Hz, ArH), 7.83 (1 H, d, *J* 7 Hz, AcN*H*Cys), 7.94 (2 H, d, *J* 8 Hz, ArH), and 9.28 (1 H, t, *J* 6 Hz, $ArCONHCH_2$); *m/z* 352 (M^+) and 190.

N-*Acetyl*-S-(N'-*phenylureidomethyl*)*cysteine* (**15b**).—*N'*-Ethoxymethyl-*N*-phenylurea (**14a**) (970 mg, 5 mmol) was added to *N*-acetyl-L-cysteine (815 mg, 5 mmol) in trifluoroacetic acid (6 ml). The mixture was stirred at ambient temperature for 15 min before the solvent was evaporated at 2 Torr. Column chromatography of the residue (silica gel; $CHCl_3$—MeOH, 7:1) gave the *cysteine derivative* (**15b**) as a colourless gum (913 mg, 59%) which could not be crystallised. A satisfactory microanalysis could not be obtained, but the sample appeared to be pure by t.l.c. and n.m.r. analysis. $\nu_{max.}$ (liquid film) 3 150, 1 705, and 1 660 cm^{-1}; δ_H [220 MHz; $(CD_3)_2SO$] 2.10 (3 H, s, Ac), 2.89 (1 H, dd, *J* 8.5 and 13.5 Hz) and 3.07 (1 H, dd, *J* 5 and 13.5 Hz) (cysteine CH_2), 3.6 (1 H, br, CO_2H), 4.41 (1 H, d, *J* 6.5 Hz) and 4.42 (1 H, d, *J* 6.5 Hz) (NCH_2S), 4.51 (1 H, dt, *J* 5 and 8.5 Hz, cysteine α-H), 6.86 (1 H, t, *J* 6.5 Hz, $CONHCH_2S$), 6.98 (1 H, t, *J* 8 Hz, ArH), 7.31 (2 H, t, *J* 8 Hz, ArH), 7.48 (2 H, d, *J* 8 Hz, ArH), 8.32 (1 H, d, *J* 8.5 Hz, AcNH), and 8.72 (1 H, s, PhNHCO); *m/z* 311 (M^+), 149.

N-*Acetyl*-S-{N-[4,6-*bis*(*dimethylamino*)-1,3,5-*triazin*-2-*yl*]-N-*methylaminomethyl*}*cysteine* (**7**).—A mixture of aqueous formaldehyde (37% w/v; 3 ml, 37 mmol), methanol (20 ml), *N*-acetyl-L-cysteine (1.0 g, 6.1 mmol) and 2,4-bis(dimethylamino)-6-methylamino-1,3,5-triazine[16] (980 mg, 5 mmol) was stirred at 37 °C for 2 h before being cooled to 0 °C for 1 h. The precipitate was filtered off and washed with a small volume of cold methanol to give the *cysteine derivative* (**7**) as a white powder (1.30 g, 70% based on the pentamethyl melamine) which decomposed without melting at < 60 °C (Found C, 45.55; H, 6.9; N, 26.1. $C_{14}H_{25}N_7O_3S$ requires C, 45.25; H, 6.8; N, 26.4%); $\nu_{max.}$ 3 230, 1 700, and 1 610 cm^{-1}; δ_H [220 MHz; $CDCl_3$–$(CD_3)_2SO$, 2:1] 1.99 (3 H, s, Ac), 3.10 (1 H, dd, *J* 7 and 13.5 Hz, cysteine β-H), 3.12 (12 H, s, NMe_2), 3.14 (3 H, s, melamine-$NRCH_3$), 3.24 (1 H, dd, *J* 4.5 and 13.5 Hz, cysteine β-H), 3.76 (1 H, m, cysteine α-H), 4.88 (1 H, d, *J* 14 Hz) and 5.07 (1 H, d, *J* 14 Hz) (NCH_2S), and 6.92 (1 H, d, *J* 8 Hz, NH); δ_C [$(CD_3)_2SO$] 22.17, 32.00, 32.44, 35.39, 40.35, 40.79, 51.92, 164.97, 169.06, and 172.10 p.p.m. *m/z* 371.1737 ($C_{14}H_{25}N_7O_3S$ requires 371.1734) (M^+), 209 (100%).

S-[N'-(4-*Chlorophenyl*)*ureidomethyl*]*glutathione Trifluoroacetate Salt Hydrate* (**16e**).—*N*-(4-Chlorophenyl)-*N'*-methoxymethylurea (**14b**) (429 mg, 2 mmol) was added to glutathione (614 mg, 2 mmol) in trifluoroacetic acid (3 ml). The mixture was stirred for 5 min before evaporation of the solvent under reduced pressure. The oily residue was triturated with diethyl ether to give a white powder. Dissolution of this material in acetone followed by reprecipitation on addition of diethyl ether and filtration furnished the *glutathione derivative* (**16e**) (1.09 g, 88%) as a slightly hygroscopic white powder without a definite m.p. but which decomposed on gentle heating (Found: C, 38.3; H, 4.7; N, 11.0. $C_{20}H_{27}ClF_3N_5O_{10}S$ requires C, 38.6; H, 4.4; N, 11.25%); $\nu_{max.}$ 3 150, 1 705, and 1 640 cm^{-1}; δ_H [$(CD_3)_2SO$] 2.07 (2 H, m, glutamyl β-CH_2), 2.39 (2 H, m, glutamyl γ-CH_2), 2.74 (1 H, m, cysteine β-H), 3.05 (1 H, dd, *J* 4.5 and 14 Hz, cysteine β-H), 3.83 (2 H, br, glycine CH_2), 4.00 (1 H, m, glutamyl α-H), 4.36 (1 H, dd, *J* 7 and 13 Hz) and 4.44 (1 H, dd, *J* 7 and 13 Hz) (NCH_2S), 4.63 (1 H, m, cysteine α-H), 7.35 (2 H, d, *J* 8 Hz, ArH), 7.53 (2 H, d, *J* 8 Hz, ArH), and 8.4 (9 H, m, NH and OH).

S-*Formamidomethylglutathione Trifluoroacetate Salt Dihydrate* (**16a**).—This compound was prepared from *N*-hydroxymethyl formamide[17] (**9**) (150 mg, 2 mmol) and glutathione (614 mg, 2 mmol) according to the method for (**16e**) above, giving the *glutathione derivative* (**16a**) (966 mg, 94%) as a hygroscopic white solid without a definite m.p. (Found: C, 32.4; H, 5.2; N, 10.6. $C_{14}H_{25}F_3N_4O_{11}S$ requires C, 32.8; H, 4.9; N, 11.0%); $\nu_{max.}$ 3 200, 1 710, and 1 660 cm^{-1}; δ_H [220 MHz; $(CD_3)_2SO$] 2.07 (2 H, m, glutamyl β-CH_2), 2.40 (2 H, m, glutamyl γ-CH_2), 2.6—3.1 (2 H, m, cysteine CH_2), 3.85 (2 H, br s, glycine CH_2), 4.00 (1 H, m, glutamyl α-H), 4.32 (0.5 H, dd, *J* 6 and 13 Hz) and 4.38 (0.5 H, dd, *J* 6 and 13 Hz) (NCH_2S of rotamer A), 4.47 (0.5 H, d, *J* 13 Hz, NCHS of rotamer B), 4.64 (1.5 H, m, cysteine α-H and NCHS of rotamer B), 8.17 (0.5 H, s, formyl H), 8.34 (0.5 H, s, formyl H), 8.42 (5.5 H, m, NH and OH), 8.75 (0.5 H, *ca.* t, *J* 6 Hz, $HCONHCH_2$ of one rotamer), and 9.5 (2 H, br, NH and OH).

S-(4-*t-Butylbenzamidomethyl*)*glutathione Picrate* (**16b**).—*N*-Hydroxymethyl-4-t-butylbenzamide[1] (414 mg, 2 mmol) was

added to glutathione (614 mg, 2 mmol) in trifluoroacetic acid (3 ml). The mixture was stirred at ambient temperature for 10 min before the solvent was evaporated at 2 Torr. The gummy residue was dissolved in acetone (20 ml) and 2,4,6-trinitrophenol (458 mg, 2 mmol) in methanol (4 ml) was added. The solvents were evaporated from this mixture under reduced pressure. The residue was precipitated from acetone solution by addition of diethyl ether to give the *glutathione derivative* (**16b**) (1.351 g, 93%) as a slightly hygroscopic bright yellow powder without definite m.p. (Found: C, 46.0; H, 5.0; N, 13.3. $C_{28}H_{35}N_7O_{14}S$ requires C, 46.35; H, 4.85; N, 13.5%); $[\alpha]_D^{23}$ −21.7° (*c* 17.5% w/v in dimethyl sulphoxide); $\nu_{max.}$ 3 150, 1 705, 1 650, 1 515, and 1 345 cm^{-1}; δ_H [$(CD_3)_2SO$] 1.31 (9 H, s, CMe_3), 2.05 (2 H, m, glutamyl β-CH_2), 2.40 (2 H, m, glutamyl γ-CH_2), 2.78 (1 H, dd, *J* 9 and 14 Hz) and 3.10 (1 H, dd, *J* 4 and 9 Hz) (cysteine CH_2), 3.7 (5 H, br, CO_2H and RN^+H_3), 3.84 (2 H, d, *J* 5.5 Hz, glycine CH_2), 4.00 (1 H, t, *J* 7 Hz, glutamyl α-H), 4.46 (1 H, dd, *J* 6.5 and 13 Hz) and 4.57 (1 H, dd, *J* 6.5 and 13 Hz) (NCH_2S), 4.68 (1 H, dt, *J* 4 and 9 Hz, cysteine α-H), 7.57 (2 H, d, *J* 8.5 Hz, benzamide ArH), 7.89 (2 H, d, *J* 8.5 Hz, benzamide ArH), 8.35 (1 H, t, *J* 5.5 Hz, glycine NH), 8.40 (1 H, d, *J* 9 Hz, cysteine NH), 8.67 (2 H, s, picrate ArH), and 9.16 (1 H, t, *J* 6.5 Hz, ArCONH).

S-Benzamidomethylglutathione Trifluoroacetate Salt Hydrate (**16c**).—*N*-Hydroxymethylbenzamide[1] (302 mg, 2 mmol) and glutathione (614 mg, 2 mmol) were treated as for the preparation of (**16e**). This method afforded the *glutathione derivative* (**16c**) (1.929 g, 90%) as a hygroscopic white powder without definite m.p. (Found: C, 40.7; H, 4.6; N, 9.2. $C_{20}H_{29}F_3N_4O_{11}S$ requires C, 40.7; H, 4.95; N, 9.5%); $[\alpha]_D^{23}$ −16.2° (*c* 39% w/v in water); $\nu_{max.}$ 3 200 and 1 690 cm^{-1}; δ_H [220 MHz; $(CD_3)_2SO$] 2.06 (2 H, m, glutamyl β-CH_2), 2.38 (1 H, dt, *J* 14 and 7 Hz), and 2.43 (1 H, dt, *J* 14 and 7 Hz) (glutamyl γ-CH_2), 2.89 (1 H, dd, *J* 10.5 and 14 Hz) and 3.11 (1 H, dd, *J* 4 and 14 Hz) (cysteine CH_2), 3.83 (2 H, d, *J* 7 Hz, glycine CH_2), 4.01 (1 H, m, glutamyl α-H), 4.48 (1 H, dd, *J* 6 and 13.5 Hz) and 4.55 (1 H, dd, *J* 6 and 13.5 Hz) (NCH_2S), 4.69 (1 H, ddd, *J* 4, 6, and 10.5 Hz, cysteine α-H), 6.0 (1 H, br, NH or OH), 7.57 (2 H, t, *J* 7 Hz, ArH), 7.62 (1 H, t, *J* 7 Hz, ArH), 7.94 (2 H, d, *J* 7 Hz, ArH), 8.38 (6 H, m, NH ± OH), and 9.24 (1 H, t, *J* 6 Hz, PhCONH).

S-(N'-Phenylureidomethyl)glutathione Trifluoroacetate Salt Dihydrate (**16d**).—This compound was prepared from *N*-ethoxymethyl-*N'*-phenylurea (**14a**) (388 mg, 2 mmol) and glutathione according to the method for (**16e**) above. The *glutathione derivative* (**16d**) (1.031 g, 90%) was obtained as a slightly hygroscopic white powder without a definite m.p. (Found: C, 39.8; H, 4.7; N, 11.5. $C_{20}H_{30}F_3N_5O_{11}$ requires C, 39.65; H, 5.0; N, 11.55%); $[\alpha]_D^{23}$ −31.0° (*c* 25% w/v in water); $\nu_{max.}$ 3 150 and 1 685 cm^{-1}; δ_H [220 MHz; $(CD_3)_2SO$] 2.08 (3 H, m, glutamyl β-CH_2 and cysteine NH), 2.40 (2 H, m, glutamyl γ-CH_2), 2.76 (1 H, dd, *J* 10 and 14 Hz) and 3.04 (1 H, dd, *J* 4 and 14 Hz) (cysteine CH_2), 3.81 (2 H, d, *J* 7 Hz, glycine CH_2), 4.01 (1 H, m, glutamyl α-H), 4.37 (1 H, dd, *J* 7 and 13.5 Hz) and 4.46 (1 H, dd, *J* 7 and 13.5 Hz) (NCH_2S), 4.46 (1 H, m, cysteine α-H), 6.99 (1 H, t, *J* 8 Hz, ArH), 7.20 (1 H, t, *J* 7 Hz, glycine NH or CON*H*CH_2S), 7.31 (2 H, t, *J* 8 Hz, ArH), 7.50 (2 H, d, *J* 8 Hz, ArH), 8.4 (5 H, br, NH and OH), and 9.02 (1 H, s, PhNH).

S-{N-[4,6-Bis(dimethylamino)-1,3,5-triazin-2-yl]-N-methylaminomethyl}glutathione Trifluoroacetate Salt.—This compound was prepared from 2,4-bis(dimethylamino)-6-(*N*-hydroxymethyl-*N*-methylamino)-1,3,5-triazine[16] (452 mg, 2 mmol) and glutathione (614 mg, 2 mmol) as for (**13e**) above; the *glutathione derivative* (520 mg, 51%) could not be purified without decomposition. ^1H N.m.r. spectroscopy indicated this white non-hygroscopic powder to be *ca.* 85% pure; $\nu_{max.}$ 3 300 and 1 660 cm^{-1}; δ_H [220 MHz; $(CD_3)_2SO$] 2.06 (2 H, m, glutamyl β-CH_2), 2.39 (2 H, m, glutamyl γ-CH_2), 2.74 (1 H, m) and 3.03 (1 H, m) (cysteine CH_2), 3.06 (12 H, s, NMe_2), 3.16 (3 H, br, s, melamine-NRC*H*$_3$), 3.80 (2 H, br s, glycine CH_2), 3.99 (1 H, m, glutamyl α-H), 4.58 (1 H, m, cysteine α-H), 4.74 (1 H, d, *J* 13 Hz) and 5.08 (1 H, d, *J* 13 Hz) (NCH_2S), and 8.4 (7 H, m, NH and OH).

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^{1}H and ^{13}C Spectra of the Rotational Isomers of *N*-Hydroxymethylamides and Derivatives

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^{1}H and ^{13}C NMR Spectra of the Rotational Isomers of *N*-Hydroxymethylamides and Derivatives†

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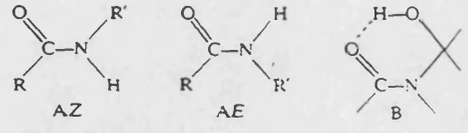
A series of *N*-hydroxymethylamides, $RCONR'CH_2OH$, and their *O*-methyl and *O*-acetyl derivatives, have been studied by ^{13}C and ^{1}H magnetic resonance spectroscopy. Signals have been assigned to the *E*- and *Z*-isomers on the basis of the analysis of the fully coupled spectra, and by comparison of the chemical shifts with those of model compounds. The introduction of the hydroxy, alkoxy or acetoxy groups at the α-position of the *N*-alkyl moiety causes a significant shift in the equilibrium towards the *E*-rotamer compared with the unsubstituted *N*-alkylamide. The predominant effect in determining the *E*:*Z* ratio appears to be the steric interaction between the carbonyl oxygen and the α-oxygen in the alkyl moiety; intramolecular hydrogen bonding does not play a significant role in determining the rotamer populations of these molecules.

INTRODUCTION

Rotational isomerism ($AZ \rightleftharpoons AE$) in *N*-alkylamides has been extensively studied using ^{13}C NMR spectroscopy[1,2] and, recently, with ^{15}N natural abundance spectroscopy.[3] In *N*-methylformamide, the *Z*-isomer is preferred by a factor of 9:1 over the *E*-isomer.[1] This present study of the ^{1}H and ^{13}C NMR spectra of a series of *N*-hydroxymethylamides, and their derivatives, was undertaken to assess the effect of the α-situated oxygen substituent on the chemical shifts in the amide and on the rotational equilibrium $AZ \rightleftharpoons AE$. The presence of a hydroxy group in R' introduces the additional possibility of hydrogen bonding, such as shown in B, which could exert a significant effect on the rotational equilibrium.



N-Hydroxymethylformamide ($HCONHCH_2OH$) has acquired special significance as a metabolite of the anti-tumour agent *N*-methylformamide (NMF).[4] In structure–activity studies of a large number of amides, NMF was found to be the most potent inhibitor of tumour growth; small changes in structure, such as substitution of the methyl with an ethyl group, result in loss of activity.[5] *N*-Hydroxymethylformamide (**1**) has been identified as a urinary metabolite of NMF[6] in mice, and although it does not possess the anti-tumour efficacy of NMF against murine tumours *in vivo*, it is active against the human mammary tumour xenograft (MX-1).[7]

Some *N*-hydroxymethylamides (carbinolamides) can be synthesized readily by reaction of the appropriate amide with formaldehyde,[8] whereas the analogous carbinolamines ($RNHCH_2OH$) usually revert spontaneously to the component alkylamine and formaldehyde.[9] Amongst *N*-hydroxymethylamides, there is often considerable variation in stability. For example, *N*-hydroxymethylbenzamide (**6**) is a stable crystalline substance, readily available from the reaction of benzamide and formaldehyde; however, *N*-methyl-*N*-hydroxymethylbenzamide ($PhCONCH_3CH_2OH$) has not been synthesized chemically, although it has been generated metabolically from *N*,*N*-dimethylbenzamide.[10] The foregoing observations suggest that all carbinolamides of structure $RCONHCH_2OH$, where R = H, alkyl or aryl, are stable; substitution of the amide NH by alkyl groups has a destabilizing effect.[11] It was of interest to examine the NMR spectra of a series of carbinolamides in order to explore whether or not intramolecular hydrogen bonding of type B is a significant factor in the geometry of these molecules and, hence, influences their stability.

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EXPERIMENTAL

Spectroscopy

Nuclear magnetic resonance spectra were obtained at 360 MHz (proton) and 90.8 MHz (^{13}C) with a Nicolet 360 NB spectrometer. The chemical shifts are referenced to TMS.

Materials

***N*-Methylformamide (9).** This was purchased from Aldrich Chemical Co. and redistilled (b.p. 65 °C at 2 Torr) before use.

***N*-Hydroxymethylformamide (1).** Obtained by reaction of formamide with paraformaldehyde according to the method of Grady and Stott.[12] Analysis: found, C 31.73, H 6.84, N 18.22; calculated for $C_2H_5NO_2$, C 32.00, H 6.71, N 18.66%.

***N*-Methoxymethylformamide (2).** Aqueous potassium hydroxide (40%) (2 ml) was added to a stirred mixture of formamide (45 g) and paraformaldehyde (33 g). After 0.5 h, methanol (200 ml) and concentrated sulphuric acid (3 ml) were added to the clear mixture, which was stirred for a further 6 h. After filtration to remove a white precipitate, dry diethyl ether (20 ml) was added to the filtrate, which was allowed to stand over sodium hydrogen carbonate overnight. After filtration, the mixture was concentrated under reduced pressure and the residue dissolved in water (50 ml) and extracted with dichloromethane (5 × 100 ml). The combined extracts were washed with water, dried and evaporated under reduced pressure to give an oil (12 g), which was distilled under reduced pressure. Three fractions were collected: (i) 1.9 g, b.p. 80–90 °C at 3 Torr; (ii) 1.7 g, b.p. 90–98 °C at 3 Torr; and (iii) 2.4 g, b.p. 98–110 °C at 3 Torr (lit.,[13] b.p. 55–58 °C at 0.1 Torr).

Fractions (i) and (ii) were combined and purified by chromatography on a silica gel (Merck, 70–230 mesh) column (60 × 1.5 cm i.d.) with chloroform as the eluent. Chloroform fractions were concentrated at room temperature under reduced pressure (2 Torr) to afford *N*-methoxymethylformamide as a colourless liquid (0.7 g). The product gave a single spot with R_F 0.5 on TLC using 5% methanol in chloroform on silica gel, developed with iron(III) chloride after conversion to the hydroxamic acid.[14] Gas chromatographic analysis of the product (1% w/v solution in acetone), injected on to a glass column (1.5 m × 4 mm i.d.) packed with 10% w/w PEGA on Chromosorb W AW DMCS (100–120 mesh) maintained at 180 °C in a Pye Unicam 204 gas chromatograph with the injector and detector maintained at 200 and 250 °C, respectively, gave a single peak with retention time 4.3 min (carrier gas, N_2 at a flow-rate of 25 ml min^{-1}) using flame-ionization detection (H_2, 45 ml min^{-1}; air, 325 ml min^{-1}).

***N*-Hydroxymethyl-*N*-methylformamide (3).** This was synthesized by the method of Grady and Stott[12] from **9** and paraformaldehyde.

***N*-Acetoxymethyl-*N*-methylformamide (4).** Crude *N*-acetoxymethyl-*N*-methylformamide was synthesized by the method of Ross *et al.*[15] The crude product (12 g) was dissolved in formamide (50 ml) and extracted with dichloromethane (3 × 150 ml). The lower layer was separated and washed with water (75 ml), dried and evaporated under reduced pressure to yield the acetate **4** as a pale yellow oil (4.7 g), free from *N*-methylformamide.

***N*-Hydroxymethylacetamide (5).** This was synthesized by the method of Milkowski *et al.*[16]

***N*-Hydroxymethylbenzamide (6).** A mixture of benzamide (20 g), formalin (50 ml) and potassium carbonate (20 g) in tetrahydrofuran (500 ml) was refluxed for 5 h, then left overnight at room temperature. The organic layer was separated from a gummy residue and the solvent evaporated under reduced pressure. The residual oil solidified slowly and was recrystallized from ethyl acetate to give hydroxymethylbenzamide. A further batch was obtained by dissolving the gummy residue in water and extracting the resulting dark red solution with ethyl acetate. The ethyl acetate extracts were washed, dried and evaporated under reduced pressure to afford the second batch of the hydroxymethylbenzamide (total yield 8.6 g, 34%), m.p. 115–117 °C (lit.,[17] m.p. 104–106 °C).

N-Hydroxymethyl-*p*-*tert*-butylbenzamide (**8**) was obtained in an analogous manner from *p*-*tert*-butylbenzamide (yield 86%), m.p. 133–134 °C (lit.,[18] m.p. 134–135 °C); $\nu_{max.}$ 3230 and 1645 cm^{-1}.

***N*-Acetoxymethylbenzamide (7).** *N*-Hydroxymethylbenzamide (5 g) and acetic anhydride (2.4 ml) were dissolved in KOH-dried, redistilled pyridine (13.3 ml) and stirred for 48 h at room temperature. The clear solution was poured over crushed ice (28 g) and left in the cold overnight. The mixture was extracted with chloroform and the extracts were washed, dried and evaporated. The residual liquid was distilled under reduced pressure to afford *N*-acetoxy-methylbenzamide (0.53 g), b.p. 150 °C at 4 Torr. (This compound has been reported[19] as the product of the lead tetraacetate oxidation of hippuric acid and, although described as a 'syrup,' no physical data have been given.)

DISCUSSION

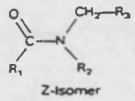
The preparation of *N*-hydroxymethylformamide (**1**) has been described.[12] The reaction of formamide with an anhydrous polymer of formaldehyde, preferably paraformaldehyde, in the presence of an alkali metal hydroxide as catalyst, is reported[12] to give 'an almost water-white pure product in 98% yield.' We have repeated this procedure, and also obtained a high yield of a colourless liquid that gave a C,H,N analysis in

agreement with the formula of hydroxymethylformamide, $C_2H_5NO_2$. However, such an analysis could also arise from a variety of mixtures, e.g. (a) a 1:1 mixture of formamide and formaldehyde, (b) a 1:1 mixture of methylenebisformamide $[(HCONH)_2CH_2]$ and bishydroxymethylformamide $[HCON(CH_2OH)_2]$ or (c) a mixture containing all of these species in addition to **1**. Indeed, the ^1H NMR spectrum of a sample of **1** prepared by Grady and Stott's method indicates that it is not pure *N*-hydroxymethylformamide. Further, the ^{13}C spectrum of this material is exceedingly complex and shows eight signals in the carbonyl-carbon region of the spectrum, *ca* 166–172 ppm. Attempts to purify the hydroxymethylformamide by chromatography or fractional distillation were unsuccessful. Clearly, spectral analysis of this complex mixture is not possible without reference to model compounds.

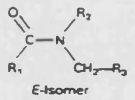
N-Methoxymethylformamide (**2**), which is a suitable model compound, was obtained in a pure form by distillation and chromatography, and a complete ^1H and ^{13}C NMR spectral analysis is possible. The ^1H NMR spectrum of **2** in D_2O clearly shows two rotational isomers. The major rotamer (*ca* 60%) is the *Z*-isomer (**2***Z*), which gives rise to signals at 8.32 (1 H, s, formyl CH), 4.72 (2 H, d, *J* 7.2 Hz, CH_2) and 3.36 (3 H, s, OMe) ppm. The minor rotamer (**2***E*) gives proton signals at 8.20 (1 H, d, *J* 11.5 Hz, formyl CH), 4.61 (2 H, d, *J* 7.2 Hz, CH_2) and 3.32 (3 H, s, OMe) ppm. The NH signals are broadened at *ca* 6.0 and 5.7 ppm. The major difference between the spectra of the rotamers is in the coupling of the formyl CH and the NH protons. The *trans* coupling constant of 11.5 Hz in **2***E* is close to the expected value for monosubstituted formamides.[1] The *cis* coupling constant is invariably lower in the *Z*-isomer (*ca* 2.0 Hz in formamides), and the apparent absence of *cis* coupling in **2***Z* is probably attributable to the electron-withdrawing effect of the $—CH_2OCH_3$ fragment (Taft σ^* +0.64), which is *trans*-coplanar to the formyl proton in **2***Z*.

The ^{13}C NMR spectrum of **2** confirms the presence of the rotational isomers **2***Z* and **2***E* in both D_2O and $CDCl_3$ solutions. In the fully decoupled spectrum in deuteriochloroform (see Table 1), the major isomer **2***Z* gives rise to signals at 161.74 (C=O), 69.72 (CH_2) and 56.02 (CH_3) ppm. Corresponding signals from the minor *E*-isomer **2***E* are seen at 165.28, 74.55 and 54.78 ppm. The conformations were assigned by comparison of ^{13}C–^1H coupling constants, obtained from the coupled spectrum of **2** in $CDCl_3$ (see Table 2), with those reported for the corresponding conformations of *N*-methylformamide.[2] The carbonyl carbon of the *Z*-rotamer (**2***Z*) is identified by its larger $^1J(CH)$ value (195.2 Hz compared with 191.5 Hz in **2***E*) and by the equality of $^2J(CNH)$ and $^3J(CNCH)$ (4.3 Hz), resulting in the doublet of quartets pattern in the fully ^1H coupled spectrum. The corresponding absorption of the *E*-rotamer is a doublet of triplets with the $^2J(CNH)$ not resolved. The value of 6.65 Hz for $^3J(CNCH)$ in **2***E*, compared with 3.1 Hz in NMF, is an effect[20] of the electronegative methoxy substituent on the coupled system. The CH_2 absorptions of the two rotamers differ mainly in the observation of an additional coupling, probably to the NH proton, in the

Table 1. ^{13}C chemical shifts of a series of *N*-hydroxymethyl-, *N*-methoxymethyl- and *N*-acetoxymethylamides



Z-isomer



E-isomer

No.	R_1	R_2	R_3	Z C=O	Z CH_2	Z R_1	Z R_2	Z R_3	E C=O	E CH_2	E R_1	E R_2	E R_3	Solvent*
1	H	H	OH	167.28	67.25	—	—	—	168.2	73.1	—	—	—	A
2	H	H	OMe	161.74	69.72	—	—	56.02	165.28	74.55	—	—	54.8	B
				167.92	72.22	—	—	57.99	171.29	77.15	—	—	57.2	A
3	H	Me	OH	167.26	69.45	—	35.9	—	168.0	76.0	—	31.1	—	A
4	H	Me	OAc	167.9	70.7	—	36.4	22.5 174.6	168.3	76.6	—	31.6	22.6 174.7	A
5	Me	H	OH	177.2	65.23	24.7	—	—	179.6	71.1	23.9	—	—	A
6	Ph	H	OH	168.4	65.04	127.2 128.7 132.1 133.6	—	—	—	—	—	—	—	B
7	Ph	H	OAc	167.65	64.54	127.1 128.7 131.8 132.8	—	20.54 171.35	—	—	—	—	—	B
8	p-*t*-Bu-C_6H_4	H	OH	168.7	65.32	31.1 35.0 125.6 126.9 130.5 155.7	—	—	—	—	—	—	—	B
9	H	H	H	166.5	26.8	—	—	—	169.75	30.3	—	—	—	A

* Solvent: A, deuterium oxide; B, deuteriochloroform.

Table 2. ^{13}C–^{1}H coupling constants (Hz) in *N*-methoxymethylformamide (2) in $CDCl_3$

	2Z	2E
^1J(CH)(C=O)	195.2	191.5
^2J(CNH)(C=O)	4.3	*
^3J(CNCH)(C=O)	4.3	6.65
^1J(CH)(CH_2)	157.3	155.7
^3J(COCH)(CH_2)	5.4	5.4
^3J(CNCH)(CH_2)	5.4	*
^1J(CH)(CH_3)	142.1	141.9
^3J(COCH)(CH_3)	5.6	5.4

* Not resolved.

spectrum of the *Z*-isomer. The methylene carbon of the *E*-isomer is a triplet of quartets, arising from the directly bonded C–H coupling and the three-bond coupling to the *O*-methyl protons, whereas in the spectrum of 2*Z* the methylene carbon is observed as a triplet of quintets. The additional multiplicity presumably arises from coupling to the formyl proton, which is suitably situated in a *trans*-periplanar position in 2*Z*, unlike the situation in 2*E*. The signals from the *O*-methyl carbons are quartets of triplets in both isomers.

The ^{13}C spectrum of **2** in D_2O is similar to that in $CDCl_3$, showing two rotamers but with slightly modified chemical shifts for each carbon. The trend of the chemical shifts is similar in the two solvents; the carbonyl and methylene carbons of the *Z*-isomer absorb at higher field than those of the *E*-isomer. These assignments were used to assign the ^{13}C signals of the rotamers of hydroxymethylformamide (**1**) and other derivatives (Table 1). Hydroxymethylformamide is a mixture of *E*- and *Z*-isomers with a slight preference for the *E*-isomer, which has ^{13}C signals at 168.2 (C=O) and 73.1 (CH_2) ppm compared with 167.3 and 67.25 for the *Z*-isomer.

These chemical shifts are remarkably close to those observed in the rotamers of *N*-methyl-*N*-hydroxymethylformamide (3*Z* and 3*E*), which shows a further pronounced shift in equilibrium towards the *E*-isomer (approximately 87% based on the relative intensity of the carbon signals at 168.0/76.0 and 167.3/69.5 ppm). The assignment of the signals at 35.9 and 31.1 ppm to the *N*-methyl carbons of 3*Z* and 3*E*, respectively, is consistent with the general observation that *N*-methyl carbons which are *syn* to the carbonyl oxygen are invariably shielded relative to the *anti* case.[2] The assignment of the signals in the spectrum of **3** was confirmed by comparison with the spectrum of the *O*-acetate derivative (**4**), which also shows a greater than 80% preference for the *E*-isomer.

The preference for the *E*-isomer of the hydroxymethylformamides in D_2O is not shared by *N*-hydroxymethylacetamide (**5**). The major form (*ca* 90%) is the *Z*-isomer with carbon signals at 177.2 (C=O), 65.23 (CH_2) and 24.7 (CH_3) ppm; the signals of the *E*-isomer appear at 179.6, 71.1 and 23.9 ppm. The carbonyl carbon atoms are deshielded relative to the formamide analogues, which is consistent with reported observations with NMF and acetamide.[2] The proportion of *E*- and *Z*-isomers in **5** is similar to that in NMF itself; in the present work, the major (90%) *Z*-isomer of NMF (9*Z*) had carbon signals in D_2O at 166.5 (C=O) and 26.8 (CH_3) ppm, compared with the published values of 164.8 and 24.7 ppm.[2] Compound 9*E* has carbon signals at 169.75 and 30.3 ppm (published values 168.0 and 28.3 ppm). Significantly, both **5** and **9** follow the general trend that the *N*-alkyl carbon which is *syn* to the carbonyl oxygen is shielded relative to the *anti* case.

The shift to the *Z*-isomer by the introduction of bulkier groups at the R position is completed in the case of *N*-hydroxymethylbenzamide (**6**), which exists exclusively as the *Z*-isomer in $CDCl_3$. The single species (6*Z*) has a carbonyl carbon which absorbs at 168.4 ppm and a methylene carbon at 65.04 ppm, in addition to the four different aromatic signals (Table 1). The introduction of an electron-donating *tert*-butyl group in the *para*-position of **6** has no effect on the equilibrium; 8*Z* is a single species (Table 1) with chemical shifts almost identical with those of 6*Z*. The complete preference of the benzamides (**6** and **8**) for the *Z*-isomer could be an indication that intramolecular hydrogen bonding, of the type shown in B, is exerting an effect. However, the acetate derivative **7** of *N*-hydroxymethylbenzamide also exists as a single species in $CDCl_3$; the chemical shifts of the carbonyl (167.65 ppm) and methylene (64.54 ppm) carbons show that the single species is also the *Z*-isomer. This observation suggests that intramolecular hydrogen bonding is not a significant factor in determining the equilibrium between *E*- and *Z*-isomers of *N*-hydroxymethylbenzamide, and that steric factors are more important. The preference of compounds **6**, **7** and **8** for the *Z*-isomer in $CDCl_3$ would thus be a consequence of steric interaction between the aryl and hydroxymethyl groups in the *E*-isomer.

In order to determine if a strongly hydrogen-bonding solvent could cause a population change in the hydroxymethylbenzamides, spectra of **6** and **8** were recorded in DMSO-d_6. However, these spectra were consistent with the presence of a single species, 6*Z* or 8*Z*, although small differences in chemical shift were observed. For example, hydroxymethylbenzamide (**6**) gave the following chemical shifts in DMSO-d_6: 166.2 (C=O), 134.2, 131.3, 128.2, 127.2 (aromatic) and 62.9 (CH_2) ppm.

Table 3 summarizes the results of analysis of the

Table 3. Percentage distribution of *E*- and *Z*-isomers of *N*-hydroxymethylamides and derivatives from NMR measurements

Compound	Solvent	Z(%)	E(%)	Nucleus
1	D_2O	43±5	57±5	^{13}C
2	$CDCl_3$	56±4	44±4	^{13}C
	$CDCl_3$	58±5	42±5	^{1}H
	D_2O	62	38	^{1}H
3	D_2O	13±6	87±6	^{13}C
4	D_2O	17.6±0.5	82.4±0.5	^{13}C
5	D_2O	90.7±3.3	9.3±3.3	^{13}C
6	$CDCl_3$	100	—	^{13}C
7	$CDCl_3$	100	—	^{13}C
8	$CDCl_3$	100	—	^{13}C
9	D_2O	90.5±0.5	9.5±0.5	^{13}C

NMR spectra to determine the percentage distribution of *E*- and *Z*-isomers in compounds **1–9**. In order to test the reliability of such data obtained from the relative intensities of signals in the ^{13}C spectra, the methoxymethylformamide **2** was analysed by both ^{1}H and ^{13}C NMR. The results are identical, within the limits of experimental error; much of the data in Table 3 can only be obtained from ^{13}C data, as the proton spectra are less well resolved in most cases. The most significant observation is the greater preference for the *E*-isomer shown by the hydroxy-, alkoxy- and acetoxy-formamides **1–4** compared with the simple *N*-methylformamide (**9**). This trend is counter to the predicted effect of hydrogen bonding (structure B), which should favour the *Z*-isomer. The percentage of the *Z*-rotamer of *N*-methylformamide observed in this study (90.5%) is close to the reported value (92%).[21] Introduction of the OH group in **1** or the OMe group in **2** shifts the equilibrium towards the *E*-rotamer, so that an almost 1:1 mixture is observed in both cases; this observation must reflect an increase in steric interaction between the carbonyl oxygen atom and the oxygen atom in the *N*-hydroxymethyl or *N*-methoxymethyl group. Introduction of a methyl group in place of the formyl proton, as in **5**, pushes the equilibrium back towards the *Z*-rotamer (>90%); in **5** the overriding factor appears to be the steric interaction between CMe and NCH_2OH groups, which destabilizes the *E*-rotamer. This trend is completed in the hydroxymethylbenzamides (**6**, **7**, and **8**), where the steric interaction with the aryl group is expected to be more pronounced and only the *Z*-rotamer is observed. In *N*-hydroxymethyl-*N*-methylformamide (**3**), the steric factor again overrides the effect of hydrogen bonding; the preferred rotamer is the one with the smaller group *syn* to the carbonyl oxygen.

The effect of introducing the α-oxygen-containing substituent into the *N*-alkyl group of *N*-methylformamide on the rotamer distribution is significantly greater than the change brought about by simply increasing the size of the alkyl group.[22] The replacement of the methyl group in NMF by the *n*-propyl group results in a slight shift towards the *E*-rotamer (8–14%), whereas the substitution of a methyl group by a methoxymethyl group results in a more significant shift to *ca* 40% of the *E*-rotamer. Evidently, the interaction between the methoxymethyl group and the carbonyl oxygen in **2** cannot simply be explained by the size of the methoxymethyl group, and may involve an electronic repulsion not present in simple *N*-alkylformamides.

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Structural Studies on Bio-Active Compounds. Part 2. The Solid State and Solution Conformations of *N*-Methyl-2-nitroethenamine and Related Compounds

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Structural Studies on Bio-active Compounds. Part 2.[1] The Solid-state and Solution Conformations of *N*-Methyl-2-nitroethenamine and Related Compounds

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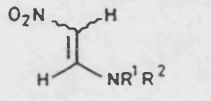
The ^{1}H n.m.r. spectra of *NN*-dimethyl-2-nitroethenamine, *N*-methyl-2-nitroethenamine, and 2-nitroethenamine in several solvents have been analysed. In the case of the monomethyl compound, the proportions of different rotamers about the C=C and C–N bonds are found to be solvent dependent. An *X*-ray crystallographic study of this compound indicates inter- rather than intra-molecular hydrogen bonding in the solid; molecular orbital calculations predict three of the possible four conformers about these bonds to be of similar and much lower energy than the fourth.

As part of a continuing study on analogues of the antitumour agent *N*-methylformamide (**2b**) (NMF), we have prepared a short series of 2-nitroethenamines (**1a**—**c**) for biological evaluation. A previous n.m.r. study undertaken by us[2] has shown (**2b**) to exhibit restricted rotation about the amide C–N bond, as for most amides, owing to delocalisation of the non-bonding pair of electrons on nitrogen into the carbonyl π-bonding system; (**2b**) shows a ratio of rotamers of *ca.* 9:1 *Z*:*E* which is almost independent of solvent. As *NN*-dimethylformamide (**2a**) and formamide (**2c**) can only exist as single 'rotamers' about this bond, it is interesting to speculate as to whether the ability of (**2b**) to change conformation has any bearing on the fact that of compounds (**2a**—**c**) only (**2b**) has significant antitumour activity in murine models,[3] has shown activity against human tumour xenografts and is currently in Phase 2 clinical trial. In this paper, we present the results of our studies on the conformations adopted by the analogous compounds (**1a**—**c**) particularly with reference to the closest analogue of NMF (**2b**), *i.e.* *N*-methyl-2-nitroethenamine (**1b**).

Rajappa[4] has recently reviewed the current state of knowledge concerning the synthesis, utility, and structure of 2-nitroenamines, stating that most examples of this class of compounds exist as the enamine tautomer with only two examples reported[5] to have the 2-nitroimine structure. Our results show that all three 2-nitroenamines (**1a**—**c**) follow the general rule in being true enamines in solution in nonacidic solvents and (**1b**) being so in the solid state.

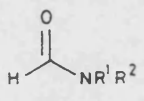
Compounds (**1a**—**c**) were synthesized generally according to known procedures. The dimethylenamine (**1a**) was obtained by condensation of dimethylformamide dimethylacetal with nitromethane.[6] The methylamino (**1b**) and amino (**1c**) compounds were prepared by transamination of *N*-methyl-*N*-phenyl-2-nitroethenamine with methylamine and ammonia, respectively.[7] Repeated recrystallisation of (**1b**) from deuterium oxide gave the N-D species (**1d**).

Two main points can be inferred from the ^{1}H n.m.r. spectra of (**1a**): geometrical isomerism about the olefinic bond and restriction of rotation about the N–C(1) bond. The C(1)H–C(2)H coupling constant is 11 Hz in both $CDCl_3$ and $(CD_3)_2SO$ solution, a value which is of little diagnostic value in determining the stereochemistry about that bond, as noted by Büchi.[8] In $(CD_3)_2SO$, the signals due to the methyl groups appear as sharp singlets at δ 2.85 and 3.20 indicating the non-equivalence reported by Büchi[8] and by Rajappa[9] for this compound. Thus, the charge-separated canonical forms (**I**)—(**III**) of (**1a**) make a contribution, *i.e.* there is significant delocalisation of the non-bonding lone pair at N(1) into the π-system of the remainder of the molecule. In $CDCl_3$, also at



(1)

a; $R^1 = R^2 = Me$
b; $R^1 = Me$, $R^2 = H$
c; $R^1 = R^2 = H$
d; $R^1 = Me$, $R^2 = D$



(2)

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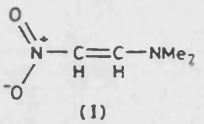
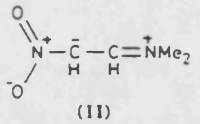


(I)



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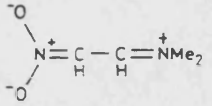

(III)

29 °C, these resonances are significantly broadened, implying that the coalescence temperature is higher in $(CD_3)_2SO$ than in $CDCl_3$. (Mannschreck[10] gives a coalescence temperature of 52 °C in $CDBr_3$.) The origins of this effect are unclear but it may arise from the greater viscosity of dimethyl sulphoxide or from such factors as the dielectric constant or dipole moment of the solvent.

Krowczynski and Kozerski[7] report a 60:40 *Z*:*E* ratio of geometrical isomers about the olefinic bond in (**1b**) in chloroform solution. We observed only one conformer to be present in solution in $CDCl_3$ at 34 °C. The magnitudes of the C(1)H–C(2)H coupling constant (5.5 Hz) and of $J_{C(1)H-NH}$ (14.0 Hz) are consistent with *Z* and *E* configurations respectively. This arrangement is stabilised by an intramolecular hydrogen bond between the NH and the nitro group, which is confirmed by the immutability of the chloroform solution i.r. spectrum upon dilution.

A mixture of three rotamers is observed in $(CD_3)_2SO$ in the ratio 23.5:7.7:68.8, as shown in Table 1. Now, three-quarters of the total population of solute adopt an *E* conformation about C(1)–C(2), reflecting the facility of hydrogen-bonding to solvent. However, of these C(1)–C(2)*E* species, the majority adopt the *Z* arrangement about the C(1)–N bond. Assign-

Table 1. Relative isomer populations of (**1b**) in various solvents

Solvent	C(1)–C(2)*Z*/C(1)–N*E*	C(1)–C(2)*E*/C(1)–N*E*	C(1)–C(2)*E*/C(1)–N*Z*	(**1ba**)	(**1bb**)
$CDCl_3$	100				
$(CD_3)_2SO$	23.5 ± 1.0	7.7 ± 0.7	68.8 ± 1.7		
D_2O	63 ± 3	37 ± 3			
CF_3CO_2H				47.5 ± 0.5	52.5 ± 0.5

Table 2. ^1H N.m.r. data for compounds (**1a—d**)

Compound	Solvent	C(1)–C(2) Stereochemistry	C(1)–N Stereochemistry	Chemical shift δ C(1)H	C(2)H	NH	CH_3
(**1a**)	$CDCl_3$	*E*		8.20 (d, *J* 11 Hz)	6.65 (d, *J* 11 Hz)		2.90br (s)
							3.25br (s)
(**1a**)	$(CD_3)_2SO$	*E*		8.30 (d, *J* 11 Hz)	6.80 (d, *J* 11 Hz)		2.85 (s)
							3.20 (s)
(**1b**)	$CDCl_3$	*Z*	*E*	6.79 (dd, *J* 14, 5.5 Hz)	6.54 (d, *J* 5.5 Hz)	9.1br	3.21 (d, *J* 5 Hz)
(**1b**)	$(CD_3)_2SO$	*Z*	*E*	7.22 (dd, *J* 15, 6 Hz)	6.48 (d, *J* 6 Hz)	9.4br	3.09 (d, *J* 6 Hz)
(**1b**)	$(CD_3)_2SO$	*E*	*E*	8.29 (d, *J* 10.5 Hz)	6.80 (d, *J* 10.5 Hz)	8.15br	3.03 (d, *J* 4.5 Hz)
(**1b**)	$(CD_3)_2SO$	*E*	*Z*	8.24 (dd, *J* 7.5, 10.5 Hz)	6.82 (d, *J* 10.5 Hz)	8.15br	2.74 (d, *J* 4.5 Hz)
(**1b**)	D_2O	*Z*		7.55	6.81 (d, *J* 6 Hz)		3.28 (s)
(**1b**)	D_2O	*E*		8.51 (d, *J* 10.5 Hz)	7.08 (d, *J* 10.5 Hz)		2.96 (s)
(**1b**)	CF_3CO_2H	Adduct (**1ba**)		5.06 (m)	5.27 (dd, *J* 5.5, 16 Hz)		3.10 (t, *J* 5.5 Hz)
					5.48 (dd, *J* 7, 16 Hz)		
(**1b**)	CF_3CO_2H	Protonated species (**1bb**)		7.90 (d, *J* 15 Hz)	8.2 (m)	8.2br	3.49 (*J* 5.5 Hz)
(**1c**)	$(CD_3)_2SO$	*Z*		7.20 (m)	6.45 (d, *J* 6 Hz)	8.1br	
(**1c**)	$(CD_3)_2SO$	*E*		6.80br (d, *J* 11 Hz)	8.25 (*J* 11 Hz)	8.1br	
(**1d**)	$(CD_3)_2SO$	*Z*	*E*	7.20 (m)	6.46 (*J* 6 Hz)		3.08 (s)
(**1d**)	$(CD_3)_2SO$	*E*	*E*	8.24 (d, *J* 10 Hz)	6.79 (*J* 10 Hz)		3.02 (s)
(**1d**)	$(CD_3)_2SO$	*E*	*Z*	8.22 (d, *J* 11 Hz)	6.83 (d, *J* 11 Hz)		2.73 (s)

ments of structure are derived from the coupling constants $J_{C(1)H,C(2)H}$ and $J_{C(1)H,NH}$ $J_{C(1)H,C(2)H}$ is 5.5 Hz for the *Z* and *ca.* 11 Hz for the *E* isomers about C(1)–C(2); values lower than those generally accepted for olefinic *cis* and *trans* vicinal coupling, owing to reduction of the double bond character in 2-nitroenamines. By analogy, the uncertainty of the stereochemistry of (**1a**) is resolved, the 11 Hz coupling being shown to be *trans*-vicinal $J_{C(1)H,C(2)NH}$. Similarly, having identified *Z* and *E* isomers about C(1)–N giving chemical shifts of δ 2.74 and 3.03 for NCH_3 protons, it is possible to assign the resonances due to the $N(CH_3)_2$ methyl protons in (**1a**) [in $(CD_3)_2SO$ solution] as δ 2.85 for the CH_3 *syn* to C(2) and as δ 3.20 *anti* to C(2). Orientation about C(1)–N was also established using coupling constant information (7.5 and 15 Hz for *Z* and *E*, respectively); Fetell and Feuer[11] give $J_{C(1),NH}$ 14 Hz for an *E*-C–N bond in a related molecule. Hence, of those molecules of (**1b**) that are *E* about C(1)–C(2), the *E*:*Z* ratio about C(1)–N is 1:8.9, a proportion that is remarkably similar to the 1:9 ratio of *E*:*Z* rotamers for the archetypal compound in our antitumour investigations, *N*-methylformamide (**2b**). Perhaps the factors involved are similar for (**1b**) and for (**2b**). In D_2O, however, owing to exchange with the solvent, the C(1)H–NH coupling is not observed and confident assignment of stereochemistry about C(1)–N is not possible. Two isomers about C(1)–C(2) are shown by $J_{C(1)H,C(2)H}$ analysis. The proportion of the *Z* (*i.e.* intramolecularly hydrogen-bonded) isomer is 63%, between the amounts present in chloroform and dimethyl sulphoxide. This reflects, perhaps, a lesser stabilisation of the *E* form (with its hydrogen-bonding to solvent) in water (dipole moment μ 1.85 D)[12] than in the dipolar aprotic dimethyl sulphoxide (μ 3.96 D).[12] Slow hydrolysis is evident in D_2O, a significant amount of products tentatively identified as methylamine and nitroacetaldehyde being present after 1 h at 34 °C.

As it was considered, in view of the D_2O spectra, that addition of D_2O to a $(CD_3)_2SO$ solution of (**1b**) might perturb the equilibria between isomers, the N–D derivative was prepared and its ^1H n.m.r. spectrum in anhydrous $(CD_3)_2SO$ was examined. Analysis of this spectrum served to confirm our assignment of the corresponding (**1b**) spectrum.

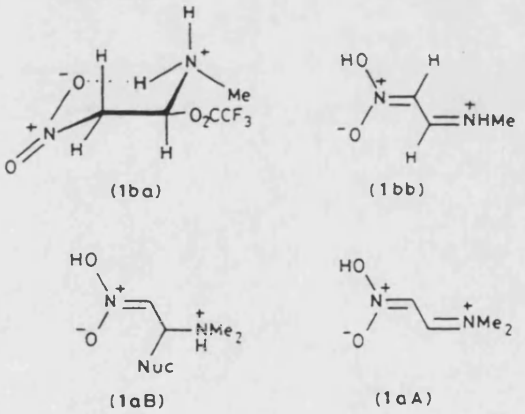

Figure 1. Proposed structures of products of (**1b**) and (**1a**) in trifluoroacetic acid. (**1ba**) and (**1bb**) are shown by n.m.r. in this study. (**1aA**) and (**1aB**) adapted from ref. 8

In trifluoroacetic acid, (**1b**) forms two distinct species in the ratio 47.5:52.5 which we denote as (**1ba**) and (**1bb**) (Figure 1). The spectrum of (**1ba**) contains an ABX system with J_{gem} of the prochiral methylene at C(2) being 16 Hz. The prochirality arises from the adjacent asymmetric centre at C(1) in the adduct (**1ba**). The N–CH_3 resonates as a triplet coupled now to two protons on N(1). Hence we put forward the structure (**1ba**) as representing this adduct. The other species appears simply to be protonated (**1b**) having the structure (**1bb**) with protonation at N(1) followed by tautomerism to give the imine nitronic acid. *E*-Stereochemistry about C(1)–C(2) can be inferred from

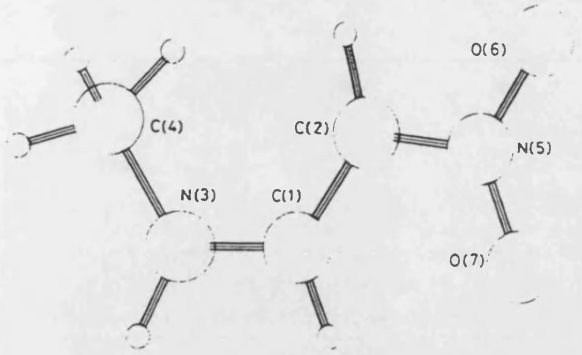

Figure 2. Structure of (**1b**) in the crystal with crystallographic numbering scheme

$J_{C(1)H,C(2)H}$ 15 Hz, clearly a *trans*-coupling constant. Büchi[8] reports the dimethyl compound (**1a**) to be unstable to aqueous acid and both he and Colonna[13] show that (**1a**) reacts with aromatic carbon nucleophiles under acidic conditions. Two intermediates (**1aA**) and (**1aB**), shown in Figure 1, are proposed in ref. 8. Whereas we concur in that (**1b**) does indeed hydrolyse very rapidly in wet trifluoroacetic acid, in anhydrous trifluoroacetic acid in the absence of a good nucleophile we observe the monomethyl analogue of (**1aA**) [*i.e.* (**1bb**)] but not that of (**1aB**). Rather, the nitro tautomer (**1ba**) of the adduct is formed.

The analysis of the i.r. spectra of (**1b**) and its deuterio-analogue (**1d**) is straightforward. In the solid phase as a KBr disc and as Nujol mull, one can observe a hydrogen-bonded N–H stretch band at 3 250 cm^{-1} which is moved to 2 370 cm^{-1} upon deuteriation (wavenumber ratio 1.37:1). A strong band is also observed at 1 615 cm^{-1}, interpretable as having contributions from C=C and C=N bonds. The spectrum in $CHCl_3$ solution was unchanged on dilution confirming the intramolecularly hydrogen-bonded structure determined by ^1H n.m.r. in $CDCl_3$ to be *Z* about C(1)–C(2) and *E* about C(1)–N. The demethyl analogue (**1c**) is virtually insoluble in chloroform; in $(CD_3)_2SO$, the ^1H n.m.r. spectrum shows it to be a mixture of the expected two stereoisomers about C(1)–C(2). The *Z* isomer ($J_{C(1)H,C(2)H}$ 6 Hz) comprises 68% of the mixture and the *E* ($J_{C(1)H,C(2)H}$ 11 Hz) represents 32%.

Crystal Structure of N-*Methyl-2-nitroethenamine* (**1b**).—The numbering scheme used in the crystallographic determination is shown in Figure 2. The crystal was grown from ethyl acetate and had dimensions 0.35 × 0.15 × 0.15 mm. The data were collected from an Enraf–Nonius CAD4 diffractometer with monochromated Mo-K_α radiation, λ = 0.710 69 Å.

Crystal data. $C_3H_6N_2O_2$, *M* = 102.09, monoclinic, *a* = 3.915(3), *b* = 12.009(7), *c* = 10.271(7) Å, β = 95.46(6)°, *V* = 480.7 $Å^3$, *Z* = 4, D_m = 1.412(5) g cm^{-3}, D_x = 1.411 g cm^{-3}, *F*(000) = 216.00, μ = 0.78 cm^{-1}, space group $P2_1/c$. Intensity data were collected by the ω–2θ scan technique. The 1 829 reflections were measured for $+h$ $\pm k$ $\pm l$ in the range $2° < \theta < 24°$ and were merged to give 760 independent reflections of which 384 were deemed observed with $F > 3\sigma$. The structure was determined by the EEES direct methods procedure in SHELX.[14] An *E*-map was produced in which all the non-hydrogen atoms were located. After least-squares refinement of positions and isotropic temperature factors, a difference Fourier synthesis located the hydrogen atoms. Further refinement of co-ordinates and anisotropic temperature factors for non-hydrogen atoms, and co-ordinates and isotropic temperature factors for hydrogen atoms, was carried out with SHELX, with the methyl C–H, H · · · H, and H · · · N distances constrained by the DFIX procedure in SHELX. In the final stages of refinement, reflections were weighted according to: $W = k/[\sigma^2(F_o) + g F_o^2] = 1.5164/[\sigma^2(F_o) + 0.000\,157\,F_o^2]$. The final discrepancy indices were $R = 0.0622$ and $R_g = \Sigma[w(|F_o| - |F_c|)^2/wF_o^2]^{\frac{1}{2}} = 0.0490$. No feature on the final difference electron density map exceeded ± 0.23 $eÅ^{-3}$.

The structure and numbering scheme[15] are presented in Figure 2 and the atomic co-ordinates and equivalent isotropic temperature factors are listed in Table 3. Anistropic thermal parameters and torsion angles are given in Supplementary Publication No. SUP 56125 (3 pp.).* Bond lengths, bond angles, and selected torsion angles are shown in Tables 4–6.

Table 3. Atom positional parameters (fractional co-ordinates × 10^4 for non-hydrogen atoms; × 10^3 for hydrogen atoms) and equivalent isotropic temperature factors (× 10^3)

Atom	*x*	*y*	*z*	U_{eq}/$Å^2$
C(1)	1 565(17)	1 746(5)	2 765(6)	48(2)
C(2)	3 130(16)	1 302(4)	3 881(5)	42(2)
C(4)	−868(21)	11(5)	1 868(8)	59(3)
N(3)	−208(13)	1 201(4)	1 834(5)	49(2)
N(5)	5 044(14)	1 971(4)	4 764(5)	50(2)
O(6)	6 331(13)	1 541(3)	5 805(4)	69(2)
O(7)	5 501(12)	2 974(3)	4 535(3)	66(2)
H(1)	178(11)	250(4)	261(4)	34(13)
H(2)	312(11)	53(3)	417(4)	32(13)
H(3)	−142(18)	161(4)	108(5)	100(23)
H(4A)	−210(15)	−20(4)	105(4)	83(24)
H(4B)	145(14)	−44(4)	187(5)	98(24)
H(4C)	−188(18)	−23(5)	272(5)	126(29)

Table 4. Bond distances for (**1b**) with standard deviations in parentheses

Bond	Distance (Å)
C(1)–C(2)	1.356(7)
C(1)–N(3)	1.303(7)
C(2)–N(5)	1.378(6)
C(4)–N(3)	1.453(7)
N(5)–O(6)	1.250(5)
N(5)–O(7)	1.243(6)

Table 5. Bond angles of (**1b**) for non-hydrogen atoms with standard deviations in parentheses

Bond	Angle (°)
N(3)–C(1)–C(2)	126.1(6)
N(5)–C(2)–C(1)	120.0(5)
C(4)–N(3)–C(1)	124.0(6)
O(6)–N(5)–C(2)	118.2(5)
O(7)–N(5)–O(6)	120.6(5)
O(7)–N(5)–C(2)	121.3(5)

Table 6. Selected torsion angles of (**1b**)

Bonds	Torsion angle (°)
N(3)–C(1)–C(2)–N(5)	177.4
N(3)–C(1)–C(2)–H(2)	−1.9
C(2)–C(1)–N(3)–C(4)	1.8
C(1)–C(2)–N(5)–O(6)	177.1
C(1)–C(2)–N(5)–O(7)	−3.2
C(1)–N(3)–C(4)–H(4A)	−176.4
C(1)–N(3)–C(4)–H(4B)	−64.6
C(1)–N(3)–C(4)–H(4C)	52.1

* For details of Supplementary Publications see Instructions for Authors in *J. Chem. Soc., Perkin Trans. 2*, 1985, Issue 1.

Table 7. Calculated ground-state energies for conformers of (**1b**)

C(1)–C(2) Stereochem.	C(1)–N Stereochem.	Electronic component (a.u.)	Nuclear component (a.u.)	Total (a.u.)	Energy above lowest isomer (kJ mol^{-1})
E	*Z*	−669.0832	298.4275	−370.6558	10.2
E	*E*	−665.3905	294.7350	−370.6555	11.0
Z	*Z*	−698.2881	327.9118	−370.3763	744.3
Z	*E*	−677.0514	306.3918	−370.6597	0

respectively. Torsion angles show that the molecule is virtually planar. Thus the amine nitrogen atom, N(3), is trigonal and this is confirmed in that the sum of the H(3)–N(3)–C(4), C(4)–N(3)–C(1), and C(1)–N(3)–H(3) bond angles is 359.7°. The nitro and methylamino groups are disposed *trans* about the carbon–carbon double bond, as reported[16] for the solid-state conformation of the dimethyl analogue (**1a**). Simple steric considerations would predict the stereochemistry about the C(1)–N(3) bond to be *E*, but it is found to be *Z* in the crystal. However, the C(1)–C(2)–H(2) bond angle is 128(2)°, bending H(2) slightly towards the nitro group and away from the *N*-methyl. Torsion angles involving the methyl hydrogens show that they are also disposed so as to relieve steric strain.

Bond lengths and angles are largely similar to those reported[16] for the dimethyl analogue (**1a**). Two main exceptions are evident; in the dimethyl compound the C(1)–N(3) bond length is 1.334(4) Å and the C(2)–N(5) distance is 1.394(4) Å. In (**1b**), these are significantly shorter at 1.303(7) and 1.378(6) Å; moreover, the C(1)–C(2) bond length is concomitantly markedly longer than a simple carbon–carbon double bond.[17] These data would indicate delocalisation throughout the $NCCNO_2$ system of (**1b**) to an even greater extent that in (**1a**). Probably there is a contribution from a charge-separated canonical form, the so-called 'push–pull' effect.

Intermolecular hydrogen bonding, as suggested by the solid-state i.r. spectra, is found to be present. Molecules in the unit cell (Figure 3), related by a screw axis, are linked by intermolecular N(3)–H(3)···O(7) bonds with an N(3)–O(7) bond distance of 2.937 Å and a N(3)–H(3)···O(7) bond angle of 164°.

Molecular Orbital Calculations for (**1b**).—Calculations of energies of the four isomers considered (*E*/*Z*, *E*/*E*, *Z*/*Z*, and *Z*/*E*) were performed by the GAUSSIAN 70 program using STO-3G. The geometry of the *E*/*Z* form was taken from the *X*-ray crystal structure determination and that of other isomers taken to be obtained by altering the appropriate torsion angles by 180°. The results are shown in Table 7. Three conformers have calculated energies within 11 kJ mol^{-1} whereas the fourth is *ca.* 744 kJ mol^{-1} higher. This prediction correlates well with the observation that, in n.m.r. experiments, judicious choice of solvent can produce these three isomers whereas we have never observed the fourth (high energy) isomer.

In two parameters, we can now test the analogy between (**1b**) and the antitumour archetype (**2b**): populations of the rotamers about the C(1)–N bond and the bond order of that bond with respect to the amide or 'pro-amide' C(1)–N bond. First, as previously noted, there is observed a 9:1 preference of *Z* over *E* for both compounds about C(1)–N as indicated by n.m.r. This is corroborated by a calculated energy difference for this isomerism of 0.8 kJ mol^{-1} in the case of (**1b**), difference of 1.3 kJ mol^{-1} calculated using STO-3G for (**2b**).[18] Secondly, comparison of the C(1)–N bond lengths determined here for the crystal of (**1b**) and calculated[18] for (**2b**) (1.303 and 1.405 Å respectively) shows that the double bond character of this formal single σ-bond is much greater in the nitroethenamine than in the formamide (**2b**).

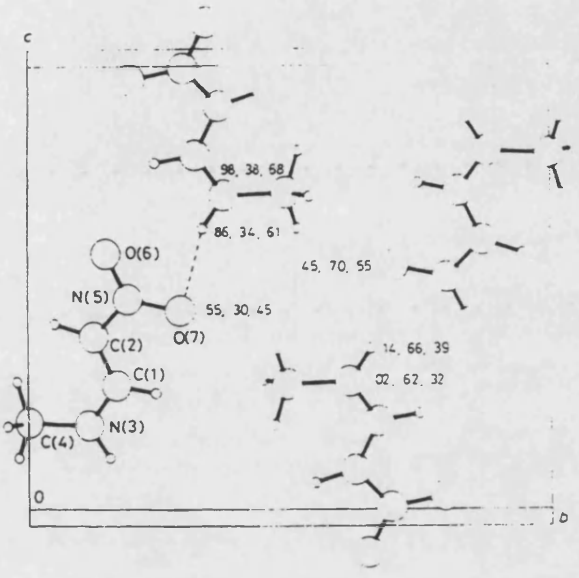

Figure 3. The unit cell of a crystal of (**1b**)

Experimental

^{1}H N.m.r spectra were obtained at 220 MHz using a Perkin-Elmer R34 spectrometer and at 60 MHz using a Varian EM360A instrument. M.p.s are uncorrected.

NN-*Dimethyl*-2-*nitroethenamine* (**1a**).—This material was prepared by the method of Meerwein[6] from nitromethane and *N*-(dimethoxymethyl)dimethylamine in 75% yield and had m.p. 104—105 °C (lit.,[6] 104 °C), $\nu_{max.}$(KBr) 3 100w, 3 030w, 2 920w, 2 820w, and 1 630 cm^{-1}.

N-*Methyl*-2-*nitroethenamine* (**1b**).—Transamination of *N*-methyl-2-nitro-*N*-phenylethenamine with methylamine according to the method of Krowczynski[7] gave (**1b**) in 87% yield. The orange-brown crystals had m.p. 121—123 °C (lit.,[7] 114—116 °C) (Found: C, 35.0; H, 5.8; N, 27.3. Calc. for $C_3H_6N_2O_2$: C, 35.3; H, 5.9; N, 27.4%); $\nu_{max.}$(KBr) 3 250, 3 100w, 3 040w, and 1 615 cm^{-1}; $\nu_{max.}$(Nujol) 3 250 and 1 615 cm^{-1}.

2-*Nitroethenamine* (**1c**).—N-*Methyl*-2-*nitro*-N-*phenylethenamine* was transaminated with ammonia as for (**1b**) above to give 2-nitroethenamine (**1c**) in 88% yield as orange-brown needles, m.p. 102—104 °C (lit.,[7] 101 °C); $\nu_{max.}$(KBr) 3 370, 3 150, and 1 625 cm^{-1}.

N-*Deuterio*-N-*methyl*-2-*nitroethenamine* (**1d**).—*N*-Methyl-2-nitroethenamine (150 mg, 1.5 mmol) was dissolved in warm deuterium oxide (99% +; Aldrich; 750 µl) followed by evaporation of the solvent under reduced pressure. The process was repeated and the residue was recrystallised from deuterium

oxide to give the *N*-deuterio compound (**1d**) as brown needles (100 mg, 0.97 mmol, 65%), m.p. 119—120 °C; $\nu_{max.}$(KBr) 3 100w, 3 050w, 2 370, and 1 620 cm^{-1}; $\nu_{max.}$(Nujol) 2 370 and 1 610 cm^{-1}.

Acknowledgements

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PUBLICATION 12

The Reaction of Diethanolamine with 2-Chloronitrobenzene - a Reinvestigation

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THE REACTION OF DIETHANOLAMINE WITH 2-CHLORONITROBENZENE - A REINVESTIGATION

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In a report on the reaction of 2-chloronitrobenzene (1) with diethanolamine (2), Meltsner et al[1] claim that the expected S_NAr product, N-(2-nitrophenyl)diethanolamine (3), is not formed; rather that the products are 2,2'-dichloroazobenzene (4), 2-nitrophenol (5), 2-chloroaniline (6) and 4-(2-aminophenyl)morpholine (7). Similar products in which the nitro function is reduced are also reported[2] for the corresponding reaction with ethanolamine. In this laboratory, in an attempted preparation of 2,2'-dichloroazobenzene (4) for reference purposes in photochemical studies on the antineoplastic agent 5-(3-azido-4-chlorophenyl)-6-ethylpyrimidin-2,4-diamine[3], the expected S_NAr product (3) was obtained along with other products.

On treatment of 2-chloronitrobenzene (1) with diethanolamine at 180°C for 3 hours, followed by extraction and chromatography, four

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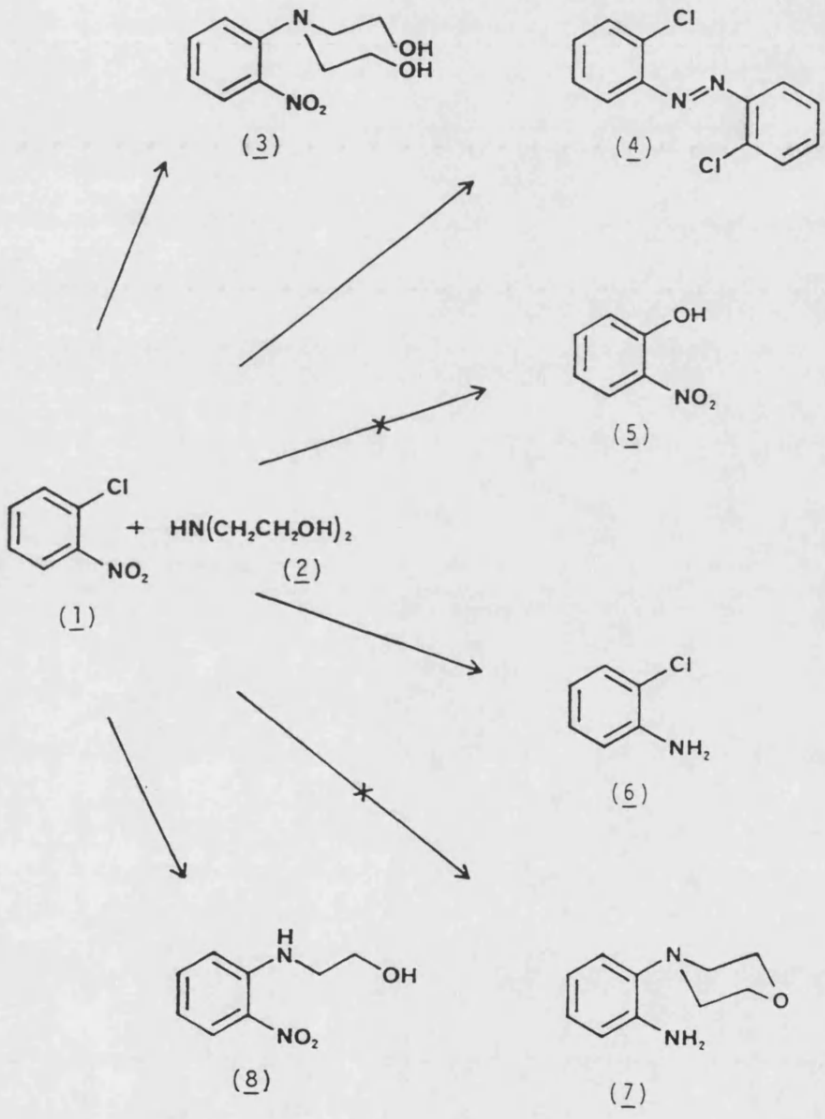
SCHEME


Cl
NO2
+ HN(CH2CH2OH)2
(1)
(2)
N
OH
OH
NO2
(3)
Cl
N=N
Cl
(4)
OH
NO2
(5)
Cl
NH2
(6)
H
N
OH
NO2
(8)
N
O
NH2
(7)

identifiable products were isolated along with a quantity of black tar. 2,2'-Dichloroazobenzene (4) (trace) and 2-chloroaniline (6) were formed as reported[1], the latter being confirmed after conversion to 2-chloroacetanilide by treatment with acetic anhydride. However, the other two claimed[1] products, 2-nitrophenol (5) and 4-(2-aminophenyl)morpholine (7), were shown by thin layer chromatography not to be present. The straightforward nucleophilic substitution product. N-(2-nitrophenyl)diethanolamine (3), was isolated in fair yield as an orange oil. The remaining major product was the monoalcohol (8) which has been prepared by Kremer[2] and by Karrer[4] from 2-aminoethanol and 2-chloronitrobenzene. This compound has already been the subject of a structure revision in Kleb's paper on the Smiles-type rearrangement of N-(2-hydroxyethyl)-2-nitrobenzenesulphonamide to (8) which was shown[5] to be identical with the "2-(2-nitrophenoxy)ethanamine" of Weddige[6]. The formation of this material could be rationalised in terms of thermal elimination of water giving an enamine. Hydrolysis by the water so generated would then afford the mono-(2-hydroxyethyl)-compound and acetaldehyde which in turn may be in part responsible for the tar formation. Interestingly, a second elimination/hydrolysis did not take place, no 2-nitroaniline being detected.

This work shows that straightforward S_NAr substitution does indeed take place between diethanolamine and 2-chloronitrobenzene, contrary to a previous report[1], and that the morpholine is not formed.

EXPERIMENTAL.

NMR Spectra were obtained in $CDCl_3$ at 60 MHz with a Varian EM360A spectrometer and IR spectra were acquired using a Perkin Elmer 1310 instrument. Melting points are uncorrected.

Reaction of 2-Chloronitrobenzene (1) with diethanolamine (2).

2-Chloronitrobenzene (10.0 g, 63.3 mmol) was heated to 180°C and diethanolamine (43 g, 405 mmol) was added during 30 min at this temperature. After being stirred at 180°C for 3 h, the mixture was cooled to 50°C and poured onto ice (150 g). The resulting dark suspension was acidified by the addition of conc. hydrochloric acid (100 ml) and extracted with dichloromethane (2 x 300 ml). The aqueous portion was basified by addition of K_2CO_3 and extracted with further quantities of dichloromethane (3 x 250 ml). Treatment with diatomaceous earth and anhydrous sodium sulphate followed by filtration served to remove suspended tar and to dry the combined extracts. Evaporation of the solvents under reduced pressure gave a dark oil. Column chromatography (silica gel, $CHCl_3$ with added MeOH increasing from 0% to 10%) afforded four compounds. In order of increasing polarity, the following were obtained 2,2'-Dichloroazobenzene (4; 50 mg, 0.6%), orange-red needles MPt 132-133°C (lit.[1], 134°C); δ 7.2-7.9 (8H, m, ArH); m/z 254/252/250 (M^+). 2-Chloroaniline (6; 3.76g, 46%), colourless liquid, ν_{max} 3470, 3380, 1615 cm^{-1}; δ 3.9 (2H, br, NH_2), 6.45-7.3 (4H, m, Ar-H); m/z 129/127, 102/100, 92; MPt of derived 2'-chloroacetanilide 86-88°C (lit.[7], 87-88°C).

2-(2-Nitrophenylamino)ethanol (8); 530mg, 4.6%), orange needles MPt 74-75°C (lit.[5], 75°C); ν_{max} 3400, 3220, 1510, 1340 cm^{-1}; δ 2.75 (2H, br, OH), 3.50 (2H, q, J 6 Hz, NCH_2), 3.90 (2H, t, J 6 Hz, CH_2OH), 6.60 (1H, dt, J 1 Hz, 8 Hz, 4-H), 6.83 (1H, dd, J 8 Hz, 1 Hz, 6-H), 7.49 (1H, dt, J 2 Hz, 8 Hz, 5-H), 8.10 (1H, dd, J 8 Hz, 2 Hz, 3-H), 8.2 (1H, br, NH); m/z 182 (M^+), 151.

N-(2-Nitrophenyl)diethanolamine (3; 1.60g, 11%), orange-brown oil, ν_{max}3380, 1595, 1510, 1340 cm^{-1}; δ 3.30 (4H, approx. t, J 4 Hz, NCH_2), 3.55 (4H, approx. t, J 4 Hz, CH_2O), 3.60 (2H, br, OH), 6.9-7.8 (4H, m, Ar-H); m/z 226 (M^+), 195 (100%), 165, 151.

ACKNOWLEDGEMENT

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The Generation of Potentially Toxic Reactive Iminium ions from the Oxidative Metabolism of *N*-Alkyl Compounds

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COMMENTARY

THE GENERATION OF POTENTIALLY TOXIC, REACTIVE IMINIUM IONS FROM THE OXIDATIVE METABOLISM OF XENOBIOTIC N-ALKYL COMPOUNDS

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During the past 30 years, a common mechanistic feature has emanated from the many investigations into mechanisms by which chemically innocuous drugs and environmental chemicals exert their toxicity. This is that they undergo metabolic activation to chemically reactive species capable of causing toxicity in specific tissues. Reports that the metabolism of the anaesthetic and psychotropic drug phencyclidine gives rise to an electrophilic iminium species [1, 2] are amongst a number of recent studies which suggest that certain alkylamines and alkylamides may have to be included in the vast array of such chemicals with hazardous potential. In this review, we summarise some of these findings and draw attention to those molecular features of alkylamines and alkylamides which favour the generation of potential toxins. Additionally, some preliminary results are presented which suggest that phase 2 metabolic reactions of metabolites of alkylamines, such as the conjugation of N-(1-hydroxymethyl) compounds, may also give rise to potentially toxic species.

The oxidative metabolism of N-alkylamines (1, Fig. 1) to N-(1-hydroxyalkyl) amines (2) is a ubiquitous metabolic pathway for many classes of xenobiotics bearing an N-alkyl group. This oxidation may be the prelude to the formation of an aldehyde, for example formaldehyde from an N-methyl compound. Alternatively, a relatively stable N-(1-hydroxyalkyl) species may be formed. The fate of this latter moiety is the focus of attention of this review and, in particular, the question is addressed of which molecular feature(s) of specific xenobiotic N-(1-hydroxyalkyl) amines may dispose them to be in equilibrium with the reactive iminium species formed by loss of hydroxide.

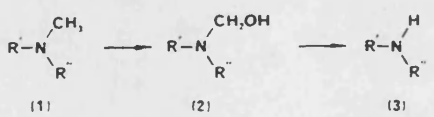

Fig. 1. Metabolism of N-methyl compounds.

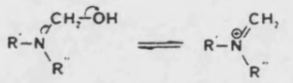

Fig. 2. Equilibrium between a carbinolamine (2) and iminium ions (4).

Several examples of the equilibrium between a carbinolamine (2, Fig. 2) and an iminium species (4) in biological systems have now been reported. The first convincing evidence for the presence of such an equilibrium was presented for a metabolite of nicotine [3]. However, there are other examples of oxidative N-dealkylation reactions where such an equilibrium does not exist, one such being that of N-(hydroxymethyl)carbazole, a metabolite of *N*-methylcarbazole (34, see Fig. 10). Beke [4] has reviewed the chemistry of the heterocyclic pseudo bases, where this equilibrium between carbinolamine and iminium ion exists. The basicity of the nitrogen atom in the carbinolamine (2, Fig. 2) naturally depends upon its structure; the equilibrium is influenced by (a) the electronegativity of substituents attached to the nitrogen (R′ and R″), (b) the situation of the nitrogen in cyclic or acyclic structures, and (c) the aromaticity of the ring. Electron-donating substituents on the nitrogen will tend to facilitate loss of the hydroxide anion. Conversely, electron-withdrawing groups decrease the electron density at the nitrogen atom and do not favour iminium ion production. This latter effect can lead to the cleavage of the N—C bond with concomitant loss of the aldehyde.

The first experiments to provide evidence for the metabolic formation of iminium ions from N-alkylamines were performed by Breck and Trager [5] in studies of the metabolism of lidocaine (Fig. 3). They suggested that the formation of a carbinolamine (6) and consequently an iminium ion (7) may explain the production of a cyclic species (8) from the oxidation of lidocaine (5). These authors also suggested that, if the formation of such a reactive, electrophilic intermediate (7) was a general phenomenon of N-dealkylation, then it might explain the biological responses, whether efficacious or toxic, of various

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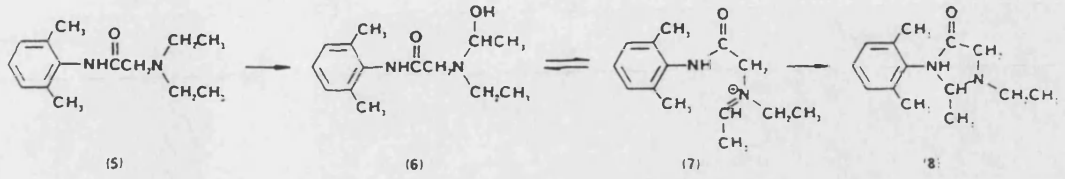


Fig. 3. Metabolism of lidocaine (5).

amine drugs, as the reaction of such intermediates with nucleophiles at critical enzyme sites [5].

In 1960, Hucker *et al.* [6], in a study of the metabolism of nicotine (9), proposed the pathway illustrated in Fig. 4. The authors studied the effect of metabolic inhibitors upon this pathway. Cyanide inhibited the production of cotinine (11) but this occurred without inhibition of the consumption of nicotine. The authors suggested that the conversion 5′-hydroxynicotine (10) to cotinine (11) was catalysed by aldehyde oxidase which had been shown to be inhibited by cyanide [7]. This suggestion was supported by evidence of the accumulation of an intermediate of metabolism formed in the presence of cyanide, which behaved like the synthetic carbinolamine (10). Murphy [3] reinvestigated these aspects of the metabolism of nicotine with the aim of elucidating the exact enzymatic steps involved in the production of cotinine. When nicotine was metabolised in the presence of cyanide, a novel compound was observed which was characterised as 5′-cyanonicotine (15, Fig. 5). Murphy proposed two pathways to be consistent with the formation of 5′-cyanonicotine (15). Pathway A (Fig. 5) involved the formation of nicotine $\Delta^{1'(5')}$ iminium ion (12) from hydroxynicotine (10), and it was considered that the ion should react readily with cyanide. Pathway B involved the formation of the aminoaldehyde tautomer (13) of hydroxynicotine, followed by reaction with cyanide (14) and ring closure in a manner analgous to the Strecker reaction. To discriminate between these two pathways, the products of the incubation of nicotine with microsomes were treated with sodium borodeuteride. This reduction yielded deuterionicotine, in which the single deuterium was located at the 5′-position (16). This observation could only be consistent with the existence of the iminium ion (12). In addition, the incubation of nicotine with microsomes under an $^{18}O_2$ atmosphere showed that the cotinine (11, Fig. 4) produced did not contain ^{18}O. It was thus demonstrated that the hydroxyl oxygen atom in the 5′-hydroxynicotine molecule was not the same oxygen as was derived from the initial enzymatic hydroxylation of nicotine, which required atmospheric oxygen. This is consistent with a pathway involving the iminium ion (12, Fig. 5).

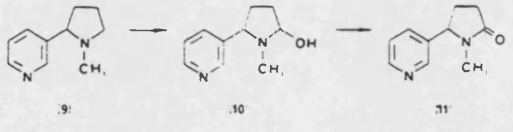


Fig. 4. Metabolism of nicotine (9) to cotinine (11).

The iminium salt of nicotine has been synthesised and its structure and stability in aqueous solution have been investigated using NMR spectroscopy [8]. In freshly prepared acid or neutral solution only the iminium form (12) was observed, whereas in strongly alkaline solutions only the carbinolamine (10) was observed. It was estimated that, at physiological pH, 25% or less was present as the carbinolamine but exact determination was not possible as the iminium ion rapidly dimerised. This finding further supports Murphy's conclusions [3] that the 5′-hydroxynicotine can exist in equilibrium with an iminium ion. The authors [8] also demonstrated that the iminium salt can be readily transformed into cotinine by means of an enzyme present in the cytosolic fraction of a liver homogenate.

Another route of nicotine metabolism is demethylation. Nguyen *et al.* [9, 10] suggested that, if nicotine (9) is demethylated via a carbinolamine (17, Fig. 6) intermediate, then an analogous iminium ion (18) might be formed and this may also be trapped by cyanide ions. Metabolic incubations were performed in the presence of cyanide and two metabolites were found; one was 5′-cyanonicotine as shown by Murphy [3], the other was determined to be *N*-(cyanomethyl)nornicotine (19). This was shown to occur, at least in part, without prior nitrogen–carbon bond cleavage, implicating the pathway illustrated in Fig. 6. Some *N*-(cyanomethyl)nornicotine appeared to be generated by an alternative pathway consisting of condensation of nornicotine (the demethylated metabolite), formaldehyde (generated during demethylation), and cyanide. However, this route appeared to be quantitatively less important.

Following the studies on nicotine, cyanide has been used as a trapping agent for iminium ions in metabolic incubations of several other agents. The tertiary amine 1-benzylpyrrolidine (20, Fig. 7) yielded several cyano adducts (21, 22, 23) which the authors proposed to arise from the nucleophilic attack by cyanide ion on a metabolically generated iminium species (24) [11]. Attack can occur at positions 2 and 5 of the pyrrolidine ring, thereby generating the dicyano adduct (22). The pyrrolidinone (23) is formed by oxidation of a hydroxylated intermediate such as (25, 26). The hepatocarcinogenic antihistamine methapyrilene (27, Fig. 8) produced only one identifiable cyano adduct, *N*-(cyanomethyl) normethapyrilene (28) [12]. The experimental evidence did not allow the authors to exclude the formation of another iminium ion (29), however, due to the chemical instability of the corresponding α-cyano amine (30). More recently the formation of an iminium ion from the widely abused drug phencyclidine (31, Fig. 9) has been studied [1, 2]. When

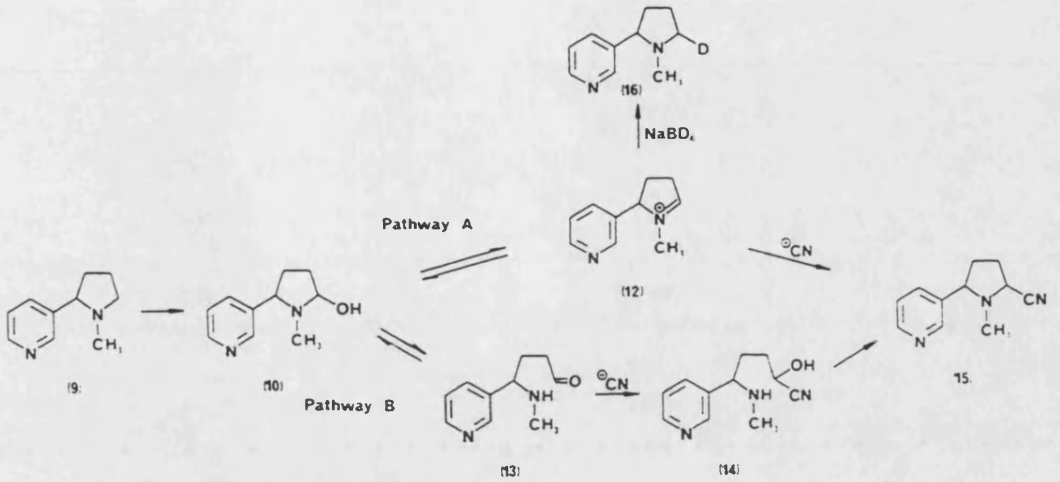


Fig. 5. Proposed pathways for the formation of 5'-cyanonicotine (15) from nicotine (9) in metabolic incubations containing cyanide.

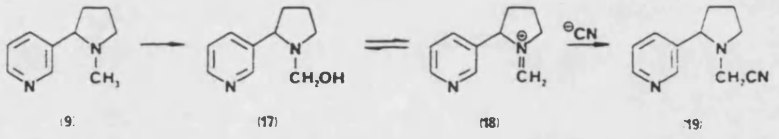
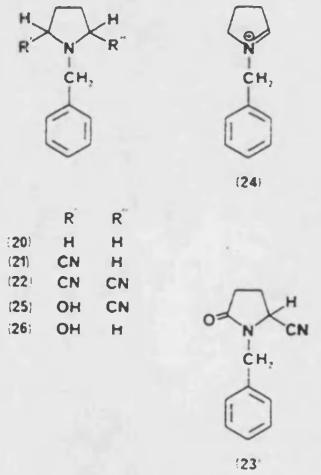
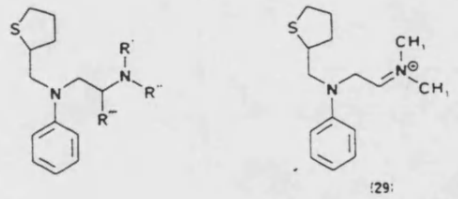


Fig. 6. Pathway for the formation of *N*-(cyanomethyl)nornicotine (19) from nicotine (9) in metabolic incubations containing cyanide.



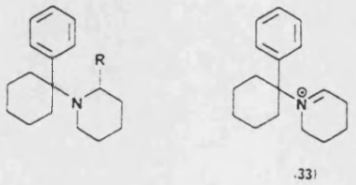
	R'	R''
(20)	H	H
(21)	CN	H
(22)	CN	CN
(25)	OH	CN
(26)	OH	H

Fig. 7. Structures of 1-benzylpyrrolidine (20) and its derivatives.



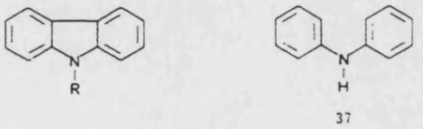
	R'	R''	R'''
(27)	CH_3	CH_3	H
(28)	CH_3	CH_2CN	H
(30)	CH_3	CH_3	CN

Fig. 8. Structures of methapyrilene (27) and its derivatives.



	R
(31)	H
(32)	CN

Fig. 9. Structures of phencyclidine (31) and its derivatives.



	R
(34)	CH_2OH
(35)	CH_3
(36)	H

Fig. 10. Structures of *N*-methylcarbazole (35), its derivatives and of diphenylamine (37).

cyanide was included in microsomal incubation of this drug, a cyano adduct was observed (32). Again, this was suggested to arise from attack of cyanide ion on an iminium ion (33). The authors also used radiolabeled drug to investigate the metabolism-dependent covalent binding of phencyclidine to microsomal protein. Cyanide, which at the concentrations used here, and in all previous experiments described, does not inhibit significantly microsomal metabolism, was a potent inhibitor of this binding, with an IC_{50} of 57 μM. Glutathione also inhibited the binding to protein. The results support the suggestion that iminium ions may be capable of reactions with nucleophilic groups on microsomal macromolecules and hence other macromolecules. Unequivocal proof of iminium ion production might better be obtained for these compounds by reduction using sodium borodeuteride.

A compound which at first sight appears to be similar to the compounds described above is N-(hydroxymethyl)carbazole (34, Fig. 10), but this compound does not form an iminium ion. Microsomal metabolism of *N*-methylcarbazole (35) under an atmosphere of $^{18}O_2$ produced N-(hydroxymethyl)-carbazole which contained ^{18}O [13]. Conversely, when the metabolism occurred in medium containing $H_2{}^{18}O$, no incorporation of ^{18}O into the N-(hydroxymethyl)carbazole was seen [13, 14]. Thus, no formation of iminium ions appeared to occur, since otherwise an exchange of the hydroxyl oxygen with that of the water would have taken place. Gorrod and Temple [15] suggested the stability of the N-(hydroxymethyl)carbazole to be due to the low basicity of the nitrogen. The nitrogen-protonated conjugate acid of *N*-methylcarbazole has a pK_a estimated to be −8 [16].

The poor basicity of *N*-methylcarbazole at the nitrogen atom and the inability of N-(hydroxymethyl)carbazole to form an iminium ion are presumably due to the involvement of the nitrogen lone pair in the aromaticity of the central ring. Protonation of the nitrogen or formation of an iminium ion would require the involvement of the nitrogen lone pair and would hence destroy this aromaticity. From comparison of the pK_a values of the conjugate acids of carbazole (36, Fig. 10) and diphenylamine (37), an estimate of 42 kJ mole^{-1} has been made for the resonance stabilisation energy derived from the central aromatic ring [16].

The proclivity of certain N-(1-hydroxyalkyl) amines to generate iminium ions appears to be dependent not only on the basicity of the nitrogen,

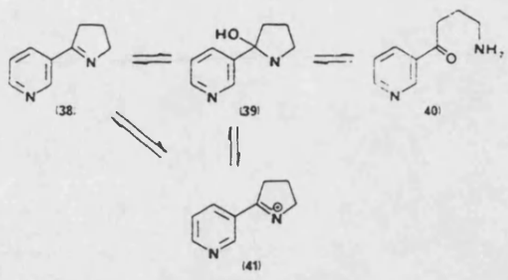
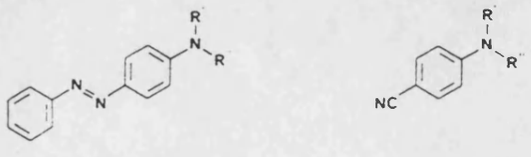


Fig. 11. Proposed equilibria for myosmine (38) in aqueous solution.

as illustrated by N-(hydroxymethyl)carbazole, but also on the extent of alkylation of the nitrogen. A recent communication [17] describes a study by nuclear magnetic resonance spectroscopy of the chemical behaviour in solution of the tobacco alkaloid myosmine (38, Fig. 11), which exists in equilibrium with the α-aminoketone (40). Resonances from myosmine (38) and the γ-aminoketone (40) were seen in the spectra of acidic solutions of myosmine, but there was no NMR evidence for the carbinolamine (39) that should, reasonably, be an intermediate in the reaction myosmine (38) $\rightleftharpoons$ γ-aminoketone (40). Evidently the life time of the carbinolamine (39) is extremely short because of the ease of dehydration to myosmine, and it is unlikely that the iminium ion (41) plays any role in this system. In the nicotine metabolite hydroxynicotine (10, Fig. 5) the presence of the N-methyl substituent prevents dehydration, and iminium ion formation is favoured.

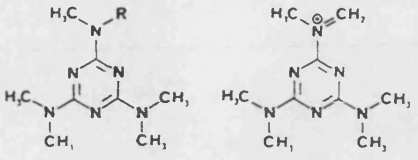
Metabolites of the following three N-methyl containing xenobiotics have recently been suggested to form iminium ions: *N,N*-dimethylaminoazobenzene (DAB) (42, Fig. 12) [18], 4-cyano-*N,N*-dimethylaniline (44, Fig. 12) [19] and hexamethylmelamine (HMM) (47, Fig. 13) [20]. A major biliary metabolite of the hepatocarcinogen DAB (42) in the rat was identified as *N*-(glutathione-*S*-methylene)-4-aminoazobenzene (43) [18], and a major urinary metabolite of 4-cyano-*N,N*-dimethylaniline (44) in the rat and mouse was shown to be *N*-acetyl-*S*-(4-cyano-anilinomethyl)cysteine (45) [19]. In both cases, the thioether metabolites were considered to be the products of the reaction between glutathione and reactive species derived from the N-hydroxymethyl compounds, presumably the methylene iminium ions



	R	R'
(42)	CH_3	CH_3
(43)	H	Glutathione

	R	R'
(44)	CH_3	CH_3
(45)	H	N-Acetylcysteine

Fig. 12. Structures of *N,N*-dimethyl-4-aminoazobenzene (42) and 4-cyano-*N,N*-dimethylaniline (44) and their metabolites.



(48)

	R
(46)	CH_2OH
(47)	CH_3
(49)	H
(50)	N-Acetylcysteine

Fig. 13. Structures of hexamethylmelamine (47) and its derivatives.

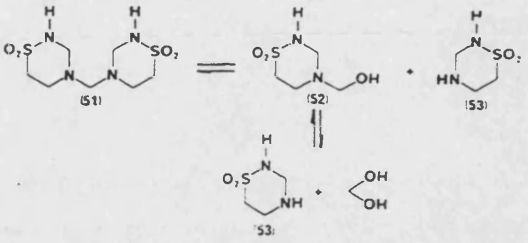


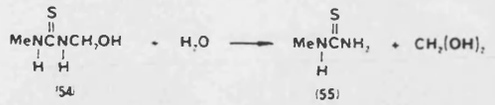
Fig. 14. Equilibria of taurolin (51) in aqueous solution.

The antineoplastic agent HMM (47, Fig. 13) is metabolized *in vitro* to *N*-(hydroxymethyl)pentamethylmelamine (HMPMM) (46) [21]. *In vitro* tests show that HMM has no cytotoxic activity *per se* but instead requires metabolic activation: HMPMM is cytotoxic and it has been suggested that this molecule might be the active cytotoxic species *in vivo* [22, 23]. It is not known, however, if HMPMM is active as an intact moiety or if it is a transport form of cytotoxic formaldehyde. Several studies have shown that the addition of semicarbazide to cell cultures protects them from formaldehyde toxicity [23–25]. The results obtained by treating cells with HMPMM after semicarbazide pretreatment are somewhat equivocal. In some cell lines, cytotoxicity of HMPMM appears to be due to formaldehyde, whereas in others it appears to be directly due to the carbinolamine. Ross *et al.* [24] compared the DNA damage caused by HMM (after metabolic activation) with that caused by formaldehyde and found only low levels of DNA–protein crosslinks with HMM, whereas non-lethal concentrations of formaldehyde caused high levels of DNA–protein crosslinks. They concluded that the cytotoxicity of HMM was unlikely to be due to formaldehyde. Studies involving [^{14}C]ring- and [^{14}C]methyl-labelled HMM *in vivo* have revealed that [^{14}C]ring-HMM binds to a high degree to cellular macromolecules, suggesting that it is the whole molecule that is bound and not only metabolites of oxidised *N*-methyl groups as would be expected if only [^{14}C]methyl-HMM was found to be bound [26]. Ames *et al.* [20] studied the binding of the two differently labelled compounds to calf thymus DNA in a microsomal incubation and found the two labels to be equally bound to the DNA. In contrast, binding of [^{14}C]methyl-HMM to protein was greater than the binding of [^{14}C]ring-HMM which may be explained by assuming that two species are involved in covalent binding: the greater binding of the [^{14}C]methyl-HMM perhaps being due to oxidative metabolism of the N-methyl groups to produce formaldehyde which consequently reacts with proteins. [^{14}C]Ring-HMPMM was shown to bind to DNA and to a lesser extent to protein. However, in this case metabolic activation was not required to obtain binding. On the basis of these results, the formation of an iminium ion (48, Fig. 13) has been proposed by Ames *et al.* [20] to explain the binding of HMPMM to cellular macromolecules, including DNA.

Studies in our own laboratories support this hypothesis. Reduction of HMPMM with cyanoborohydride produced a small quantity of HMM, the major product being pentamethylmelamine (49) from base-catalysed decomposition of HMPMM.* The HMM produced can only result from the reduction of the methylene iminium ion suggested by Ames *et al.* [20]. Furthermore, incubation of pentamethylmelamine, formaldehyde and *N*-acetylcysteine yielded a *N*-acetylcysteine conjugate of HMM (50) [27]. The mechanism of this reaction is not clear, but one possibility is an initial reaction between formaldehyde and PMM to form the carbinolamine HMPMM, which subsequently forms an iminium ion which reacts with *N*-acetylcysteine.

The question as to whether carbinolamines and carbinolamides exert their toxicity via formaldehyde or iminium ions has been investigated recently by Gidley *et al.* [28] with respect to the mode of action of the "masked" formaldehyde antibacterial agent taurolin (51). In aqueous solution the equilibrium is established between (51), the carbinolamine (52), the thiadiazine (53) and formaldehyde as shown in Fig. 14. The amounts of formaldehyde produced in this equilibrium appeared to be insufficient to explain the antibacterial activity of taurolin, and the carbinolamine (52) was implicated as the active moiety. To test this hypothesis, other carbinolamines were generated *in situ* by mixing aqueous solutions of simple amines and formaldehyde [28]. The authors investigated whether it was possible to correlate antibacterial activity with the presence of methylene iminium ions. Iminium ions were detected by adding to the reaction mixture sodium cyanoborohydride which reduces the iminium ion to the corresponding N-methyl compound while only slowly reducing formaldehyde to methanol. The reaction was followed by NMR spectroscopy. Solutions that demonstrated the presence of iminium ions also showed antibacterial activity, whereas those mixtures that did not generate iminium ions, even if they could form carbinolamines, showed only low levels of antibacterial activity. In contrast, the antibacterial agent noxythiolin (54, Fig. 15) decomposes to give only formaldehyde and the amide (55) [29]. Here, treatment with sodium cyanoborohydride yielded methanol with no indication of the presence of an iminium ion. In addition, for different concentrations and ages of the solutions, the antibacterial activity of

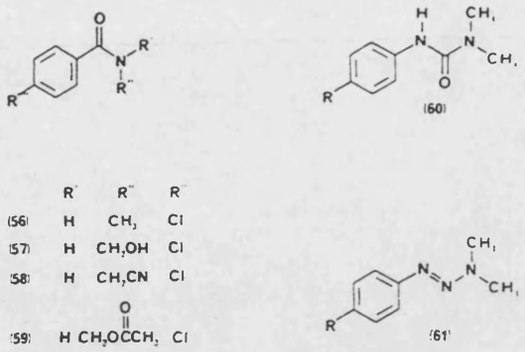
* Unpublished observation.



(54) (55)

Fig. 15. Equilibrium of noxythiolin (54) in aqueous solution.

noxythiolin solutions was very similar to that of the amounts of free formadehyde present. These results suggest that formaldehyde is the active antibacterial agent. The authors explained the observations obtained with the different "masked" formaldehyde antibacterials in terms of the chemical properties of the N-CH_2-X group. In cases where X is a reasonably good leaving group and the nitrogen possesses a basic non-bonding electron pair, formation of an iminium ion may occur. However, when X is OH and it is not easily expelled because the nitrogen is not basic, then the N-CH_2-OH moiety may break down differently to give formaldehyde which, in the absence of other nucleophiles, is rapidly hydrated. Investigations in our own laboratories on a number of N-methyl and N-hydroxymethyl compounds are in accordance with this interpretation. We [30, 31] and others [32] found relatively stable N-hydroxymethyl compounds as metabolites of N-methyl compounds when the nitrogen was in the chemical environment causing low basicity, such as in amides (56), triazenes (61) and ureas (60, Fig. 16). Not surprisingly, the loss of hydroxide from these stable N-hydroxymethyl compounds is not favoured, so that, for example, 4-chloro-*N*-(hydroxymethyl)benzamide (57, Fig. 16), a metabolite of 4-chloro-*N*-methylbenzamide (56) [31], did not react with cyanide.* However, the possibility exists that the generation of iminium ions from N-alkyl compounds of low basicity may come not directly from oxidation of the N-alkyl moiety as in the case of, for example, nicotine, but instead after conjugation of the hydroxyalkylamine or hydroxyalkylamide. Indeed, we found that the acetate esters of certain carbinolamides can react to form cyanide adducts. Incubation of *N*-(acetoxymethyl)-benzamide (59) with cyanide produced 4-chloro-*N*-(cyanomethyl)benzamide (58).* An equally striking example of how phase 2 metabolism might activate an otherwise unreactive *N*-hydroxymethyl com-



	R'	R''	R'''
(56)	H	CH_3	Cl
(57)	H	CH_2OH	Cl
(58)	H	CH_2CN	Cl
(59)	H	CH_2OCCH_3	Cl

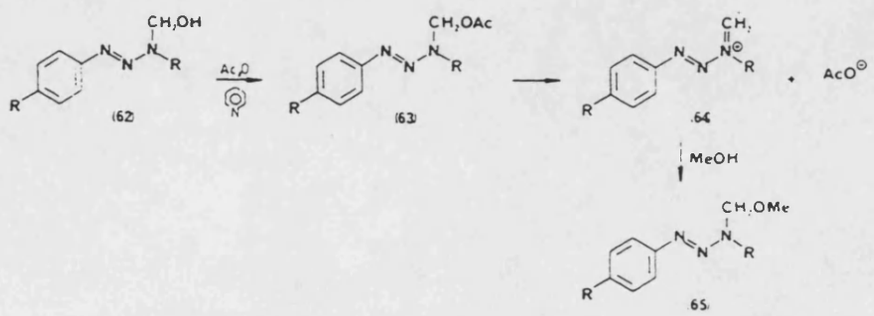
(60) (61)

Fig. 16. Structures of 4-chloro-*N*-methylbenzamide (56), its derivatives, *N*,*N*-dimethylphenylurea (60) and *N*,*N*-dimethylaryltriazene (61).

pound is found in the solvolysis reactions of some recently reported *N*-(hydroxymethyl)triazene derivatives [33]. The *N*-(acetoxymethyl)triazenes (63, Fig. 17) are readily obtained by base catalysed acetylation of the *N*-(hydroxymethyl)triazenes (62). The acetates (63) undergo solvolysis to the *N*-(methoxymethyl)triazenes (65) perhaps via the intermediate iminium ion (64) in methanol, whereas the underivatised (unconjugated) *N*-(hydroxymethyl)triazene (62) does not generate iminium ions under the same conditions. The only reaction of *N*-(hydroxymethyl)triazenes in methanol is the loss of formaldehyde to produce the monomethyltriazene (Ar-N=N-NHR).

In conclusion, in this review we have summarised the reports which have shown that reactive, electrophilic, iminium ions may be generated by the metabolism of a variety of N-alkylamines and N-alkylamides. The ability of these compounds to form such species is dependent upon their basicity. In the case of hexamethylmelamine (47, Fig. 13) the generation of an iminium ion during its metabolic N-demethylation is suggested to lead to a compound capable of reaction with DNA [20]. This result raises the question of whether this and other xenobiotics which have the potential to form an iminium ion may therefore be genotoxic, a question presently under investigation by us. In addition, such agents may prove to be antitumour agents. The biological activity of these compounds is largely undefined and

* Unpublished observation.



(62) (63) (64) (65)

Fig. 17. Chemical formation of an *N*-(acetoxymethyl)triazene (63) and its reaction with methanol.

research on them may usefully provide a wealth of information in the future on both their hazards and utility.

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PUBLICATION 14

Identification by Proton NMR of *N*-(Hydroxymethyl)-*N*-methylformamide as the Major Urinary Metabolite of *N,N*-Dimethylformamide in Mice

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IDENTIFICATION BY PROTON NMR OF N-(HYDROXYMETHYL)-N-METHYLFORMAMIDE AS THE MAJOR URINARY METABOLITE OF N,N-DIMETHYLFORMAMIDE IN MICE*

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Summary

Urine samples from mice which had received N,N-dimethylformamide were investigated by high field ^{1}H-NMR spectroscopy. The most prominent signals in the N-CH_3 region had chemical shifts identical with those of N,N-dimethylformamide (δ 2.85, 3.01) and N-(hydroxymethyl)-N-methylformamide (δ 2.91, 3.05). Resonances downfield of δ7.5 (from formyl protons) also coincided with those of the reference formamides. When [^{14}C]methyl-labelled N,N-dimethylformamide was injected and urine samples investigated by radio thin layer chromatography, the major area of radioactivity corresponded to the Rf of N-(hydroxymethyl)-N-methylformamide. Dimethylamine and methylamine were found to be minor metabolites of N,N-dimethylformamide.

The industrial solvent DMF (Fig. 1) which shows some antineoplastic effects in mice (2) and is an hepatotoxin (3-6), undergoes extensive metabolism in laboratory animals (7) and in workers who are occupationally exposed to its vapor (8). The N-desmethyl analogue NMF (Fig. 1), an investigational antitumor drug (9,10) and a potent hepatotoxin (11,12), has been considered to be the major urinary metabolite of DMF (7,8). Recently, however, it has been suggested (13,14) that the metabolite previously taken to be NMF on the basis of GLC analysis (15) is not NMF but HMMF (Fig. 1) which is thermally labile giving NMF and formaldehyde. Yet, conclusive analytical evidence to support this latter contention has hitherto been lacking. Here we

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*Part7 in the series: The formation and metabolism of N-hydroxymethyl compounds; for part 6 see ref. (1).

+To whom correspondence should be addressed.

Abbreviations: DMF, N,N-dimethylformamide; DNBS, 2,4-dinitrobenzenesulfonic acid; GLC, gas liquid chromatography; HMMF, N-(hydroxymethyl)-N-methylformamide; NMF, N-methylformamide; NMR, nuclear magnetic resonance; TLC, thin layer chromatography.

present unambiguous evidence from high-field ^{1}H-NMR spectroscopy that HMMF is indeed the major urinary metabolite of DMF. Additionally, we characterize two minor in vivo metabolites of DMF as dimethylamine and methylamine, which are conceivably enzymatic hydrolysis products of DMF and NMF respectively. The usefulness of high-resolution high-field NMR spectroscopy in the analysis of urinary metabolites of drugs has recently been described for acetaminophen (16). This report presents another example of the important role of NMR spectroscopy in urinary metabolite identification.

Materials and Methods

DMF (Aldrich Chemical Co, Gillingham, Dorset, UK), $^{2}H_7$-DMF (Sigma Chemical Co, Poole, UK) or [^{14}C]methyl-DMF (a gift from C. Steffen, Marburg, W. Germany; 96% pure) were given intraperitoneally (400 mg/kg in 0.2 ml saline) to CBA/CA mice (20-25 g). Animals were kept in metabolism cages for 24 hr and urine samples were collected and kept at -20 °C until analysis. Spectra of urine samples and reference compounds in D_2O at concentrations <1% were obtained using a Bruker WH400 instrument. Sodium 3-(trimethylsilyl)-propan-1-sulfonate was used as internal marker. Suppression of the H_2O signal in the vicinity of δ4.8 was achieved by selective presaturation (1.5s) followed by collection of free induction decay, with 4 preliminary dummy scans. Water suppression affected resonance signals caused by N-hydroxymethyl methylene protons in HMMF which could therefore not be observed in urine. HMMF was prepared by E N Gate according to Grady and Stott (17). Signals observed upfield of δ4.0 and downfield of δ7.0 were suitable for analytical diagnosis and were compared with signals in spectra of reference compounds. The urine signals recorded in table 1, column 5, were obtained from a spectrum of a sample of pooled urine of 4 female mice. Urine of DMF-treated male mice afforded an almost identical spectrum.

For TLC analysis urine samples were placed on silica gel 60 F254 plates (Merck AG, Darmstadt, W. Germany) of either 0.2 or 2 mm coat thickness, which were developed in chloroform:methanol 4:1 (v/v). Location and semiquantitative TLC autoradiography of radiolabelled metabolites were performed by methods described previously for [^{14}C]NMF (18). The spray reagent for the detection of formaldehyde precursors was a mixture of phenylhydrazine and iron (III) chloride (19). Treatment of urine samples with DNBS and quantitation of urinary alkylamines by HPLC as their 2,4-dinitrophenyl derivatives was performed according to Baba et al (20) with modifications (18). Under these conditions appreciable evaporation of amines did not occur.

Results

^{1}H-NMR analysis of urine samples from mice which had received DMF afforded several signals which were absent from the spectra of control urine or of the urine of mice treated with $^{2}H_7$-DMF (Table 1). The most prominent signals from the urine of DMF-treated mice had chemical shifts identical to those of the methyl protons of DMF and HMMF. The integral ratio of signals at δ2.85 and δ3.01 is 1:1 corresponding to the resonances from the methyl groups of reference DMF, which are trans ($J_{\text{formyl H,CH}_3}$ 0.5 Hz) and cis ($J_{\text{formyl H,CH}_3}$ 0Hz) respectively.

The two signals for the methyl protons of HMMF at δ2.91 and δ3.05 have an integral ratio of ca. 5:1 and arise from E and Z rotamers respectively. We have previously (21) determined a ratio of 87±6:13±6 for this compound using ^{13}C-NMR spectroscopy. Assignment of rotamers was made from the spectrum of authentic HMMF in D_2O with transoid 4-bond couplings (formyl H to CH_2OD or CH_3) being observed and cisoid couplings absent. The major (E) rotamer gave

$^{3}J_{formylH,CH_2}$ 3.0 Hz and the minor (Z) species gave $^{3}J_{formylH,CH_3}$ 0.5 Hz.

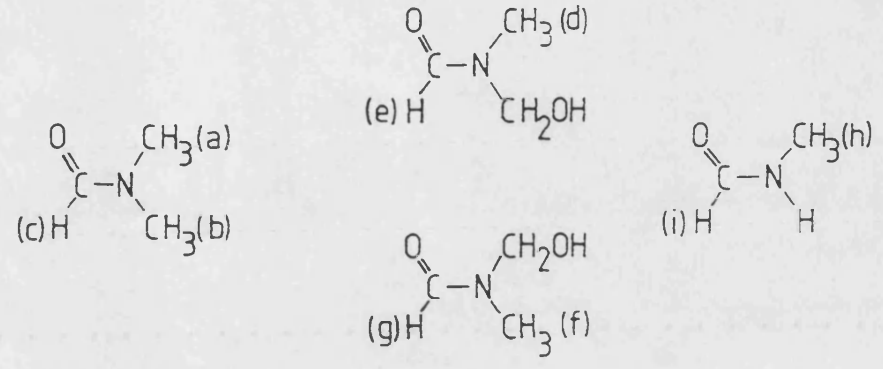

FIG. 1

Structures of DMF, HMMF and NMF.

TABLE 1

Chemical shifts of major ^{1}H-NMR signals in the spectra of control urine and urine of mice after administration of DMF or $^{2}H_7$-DMF (400 mg/kg).

Region	δ reference compounds* with ratios of relative integration	δ control urine	δ urine after $^{2}H_7$-DMF	δ urine after DMF** (integration)
δ2.5 - δ3.5	2.60 $[CH_3NH_3^+]$	2.60	2.60	2.60 (5)
	2.72 $[(CH_3)_2NH_2^+]$	2.72	2.72	2.72 (26)
	2.75 (h)			
	2.85 (b)			2.85 (19)
				2.86 (8)
	1:1	2.89		
	2.91 (d)			2.91 (64)
	3.01 (a)			3.01 (20)
	5:1	3.02	3.02	
	3:1	3.03		3.03 (8)
	3.05 (f)			3.05 (12)
	3:1			
δ7.5 - δ8.5	7.93 (c)			7.93 (6)
	8.03 (g,i)			8.03 (4)
	8.16 (e)			8.16 (21)
		8.45	8.45	8.45 (3)

* for proton assignment see Fig. 1.

** In addition there were minor signals at all chemical shifts of the control urine resonance frequencies and at δ2.75.

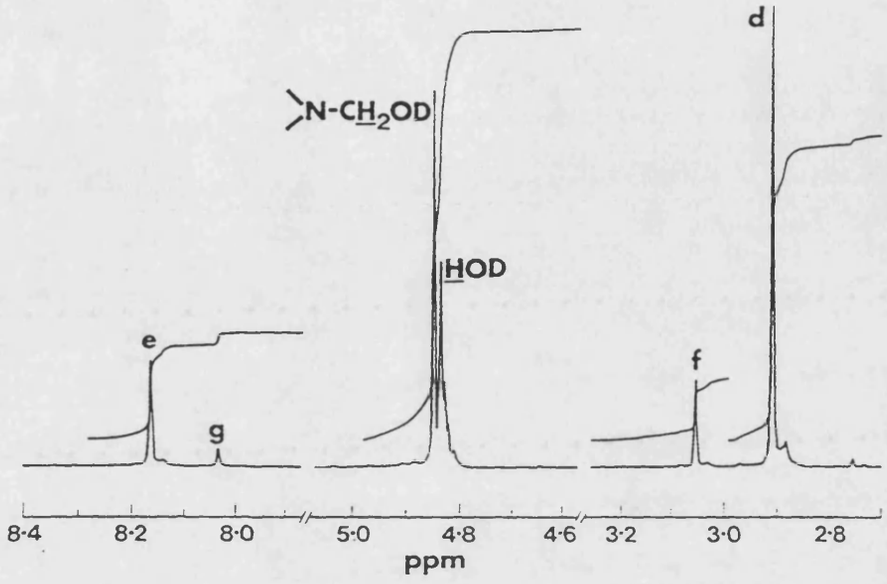

FIG. 2

^{1}H-NMR spectrum of the metabolite isolated by TLC. For assignment of signals to protons see Fig. 1.

Resonances downfield of δ 7.5 arose from the formyl protons. Assignment of these signals to the various formamides present was made on the basis of comparison of chemical shifts with reference compounds and on integration vis-a-vis the N-CH_3 signals. Only a very small peak was observed in the spectrum of urine from DMF-treated mice at δ 2.75 (corresponding to the major Z rotamer of NMF) confirming that NMF is but a minor metabolite of DMF.

Radioactivity detected on radio-TLC analysis of urine samples of mice which had received [^{14}C]methyl-DMF was concentrated in one area (Rf 0.65) representing 45% of the dose. Radioactivity was also present at Rf 0.02 (7% of the dose), which co-chromatographed with dimethyl- and methylamine, and Rf 0.85 (1% of the dose), which was the Rf value of [^{14}C]DMF. The major metabolite gave a positive colour reaction for formaldehyde. The plate material in the vicinity of Rf 0.65 was eluted with water. On GLC analysis under the conditions described previously (13) the eluted material like authentic HMMF afforded a peak identical to that produced by NMF. NMR analysis of a freeze-dried extract yielded a spectrum containing signals identical to those of authentic HMMF (Fig. 2).

NMR spectra of urine samples contained two resonance frequencies with chemical shifts identical to those of the methyl protons of dimethylamine hydrochloride (δ 2.72) and methylamine hydrochloride (δ 2.60). These protons were present in control urine and urine of $^{2}H_{7}$-DMF-treated mice but were

markedly augmented in the urine of DMF-treated mice, which indicates that both amines are DMF metabolites. HPLC analysis of urinary alkylamines after derivatization with DNBS also showed a significant increase of dimethylamine and methylamine excreted after DMF administration. In the case of dimethylamine, the amount excreted exceeded the control level by a factor of 4 and represented 3.9±1.9% (n=3) of the dose. The amount of methylamine excreted after DMF administration increased by a factor of 5 over the control level and constituted 4.1±0.7% of the dose.

Discussion

It has been suggested that the major urinary metabolite of DMF which has been characterised as NMF on GLC analysis of urine samples of animals (7) or humans (8) exposed to DMF is really HMMF and not NMF (13,14). This hypothesis has so far been based on two ambiguous pieces of evidence. The urine of DMF-treated mice has been shown to contain a stable formaldehyde precursor (13), but its chemical identity was not characterized further. Recently, evidence has been presented (22) which shows that among the constituents in the urine of rats treated with DMF was one which afforded the fragments of authentic HMMF on mass spectrometric analysis after separation by GLC. However, the definitive interpretation of the mass spectra of small molecular weight constituents in urine suffers from the scarcity of uniquely characteristic mass fragments. The results presented here show unequivocally that the major urinary metabolite of DMF in mice is indeed HMMF and not NMF.

Whereas DMF is metabolized only to a minor extent when incubated with liver preparations (13,15,23), it undergoes extensive metabolism *in vivo* (7,8,13,14). In mice 56% of a dose of 400 mg/kg was excreted as what we have now shown to be HMMF within 24 hr after administration, and only 5% as unchanged DMF (13). Dimethylamine and methylamine are also, albeit minor, metabolites of DMF in mice.

According to the results presented here, it is unlikely that NMF is responsible for the hepatotoxic potential of DMF. However, HMMF might be responsible. The biological properties of HMMF are not known and it is conceivable that it is hepatotoxic. Certain alkylamines are metabolized to carbinolamines which, by loss of hydroxide, can exist in equilibrium with reactive, potentially toxic alkylene iminium ions (24). However, on the basis of experiments which showed the lack of proclivity of N-(hydroxymethyl)-amides such as N-(hydroxymethyl)-benzamide and N-(hydroxymethyl)-formamide to form methylene iminium ions (unpublished data) we consider it unlikely that HMMF is the precursor of a toxic iminium species.

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PUBLICATION 15

Structural Studies on Bio-Active Compounds. 4. A Structure - Antitumour Activity Study on Analogues of *N*-Methylformamide

E. N. Gate, M. D. Threadgill, M. F. G. Stevens, D. Chubb, L. M. Vickers, S. P. Langdon, J. A. Hickman and A. Gescher

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Structural Studies on Bioactive Compounds. 4.[1] A Structure–Antitumor Activity Study on Analogues of *N*-Methylformamide

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A series of derivatives of *N*-methylformamide (NMF), an experimental antitumor agent, has been prepared, having the general formula $R^3C(X)NR^1R^2$ where R^1 = H, CH_3, CD_3, CH_2CF_3, CH_2CH_2Cl, cyclopropyl, C_2H_5, CH_2OH, CH_2OR, $CH_2N(CH_3)_2$; R^2 = H, CH_3; R^3 = H, CF_3, CCl_3, CH_3, Ph, $NHCH_3$, $N(CH_3)_2$; and X = O, S, NH. A further short series of "push–pull" olefins of the general formula $R^1R^2C{=}CHNR^3R^4$ has been synthesized where R^1 = H, CH_3 and R^2 = H, NO_2, CN, CHO, CH_3 and R^3 = H and R^4 = H, CH_3, morpholino. These compounds have been tested for activity against the M5076 ovarian sarcoma and the TLX5 lymphoma in mice. NMF was by far the most potent agent of both series with activity against both tumors. Some other compounds showed weak activity, but there is a rigorous structural requirement for activity and most analogues were inactive. Certain members of the series exist as equilibrium mixtures of rotamers about the amide or pro-amide bonds as shown by NMR.

The antitumor activity of *N*-methylformamide (NMF; NSC 3051: 1) in experimental use was first described[2] in 1953. A subsequent clinical trial[3] in five patients was terminated when indications of hepatotoxicity intervened. We have shown that the hepatotoxicity of NMF toward mice can be minimized if the drug is scheduled in divided doses;[4] moreover, optimum antitumor activity is elicited if the drug is administered in a chronic schedule.[4] On the basis of these preclinical studies, a new phase 1 trial was conducted, and the dose-limiting toxicities were hyperbilirubinemia, nausea, and malaise. Remarkably the agent has no myelosuppressive activity in rodents or in man.[5] Beneficial effects of 1 in combination with conventional (myelosuppressive) antitumor agents have been demonstrated against rodent experimental tumors.[6] The drug is now in phase 2 trial particularly against lung and colon tumors since the compound is very active against the NCI lung (LX-1), colon (CX-1), and mammary (MX-1) human tumor xenografts implanted in mice.[7]

Earlier studies on analogues of NMF tested against the Ehrlich ascites[8] and sarcoma 180 tumors[2] revealed that only the simplest amides, NMF, and formamide 2 had antitumor activity. We have screened a range of formamides, thioformamides, acetamides, benzamides, ureas, thioureas, guanidines, enamines, and vinylogous amides 3–35 and some related compounds 36–39 against either the TLX5 lymphoma or the M5076 reticulum cell sarcoma (or both). These tumors are sensitive to a range of agents that have an *N*-alkyl group bearing an electron-withdrawing substituent. The TLX5 lymphoma is especially sensitive to nitrosoureas,[9] triazenes,[10] and the recently discovered imidazotetrazines[11] whereas the M5076 tumor is additionally responsive to the 1,3,5-triazine series based on hexamethylmelamine.[12] Structure–activity studies in the aforementioned agents have confirmed a requirement for either an *N*-methyl or *N*-(2-haloethyl) fragment for optimum antitumor activity. It was of interest, therefore, to investigate whether or not there are similar structural

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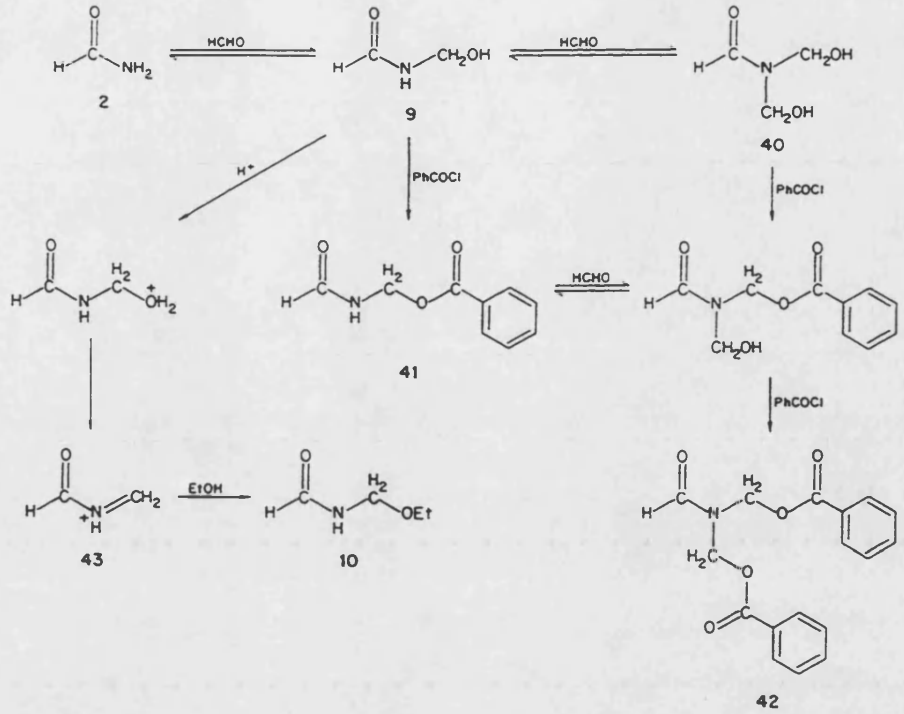
Scheme I



requirements in a series of analogues of NMF.

Chemistry

Preparation of the simple *N*-alkylformamides **3–6** and *N*-hydroxy-*N*-methylformamide (**15**) was achieved by formylation of the appropriate amine by alcoholic ethyl formate in the presence of an inorganic base. However 2,2,2-trifluoroethylamine proved to be insufficiently nucleophilic toward this agent, and a more vigorous method, previously used by us[13] for the synthesis of *N*-methyl-[^{14}C]formamide involving controlled fusion of sodium [^{14}C]formate with the amine hydrochloride, was employed for the synthesis of **7**. The isomeric *N*-methyltrifluoroacetamide (**18**) was prepared directly from methylamine and trifluoroacetic anhydride. Attempts to alkylate NMF **1** or formamide **2** at nitrogen using a variety of conditions, including the use of the alumina-supported potassium fluoride catalyst reported by Yamawaki,[14] were unsuccessful.

Hydroxymethylation of formamide and NMF with formaldehyde in the presence of a catalytic amount of base gave the carbinolamides **9** and **12**, respectively; these colorless oils were thermally unstable and could not be purified by distillation. In contrast, the carbinolamide **22** could be crystallized to purity.[15] The major contaminant in **12** (shown by ^{1}H NMR) was 5% of the starting NMF whereas the NMR spectrum (^{1}H and ^{13}C) of *N*-(hydroxymethyl)formamide (**9**) showed numerous impurities including, probably, *N*,*N*-bis(hydroxymethyl)formamide (**40**). The involvement[16] of **40** in the $HCONH_2/HCHO/HCONHCH_2OH$ system is also indicated in the reaction of **9** with benzoyl chloride in which both the expected monoester **41** and bis[(benzoyloxy)methyl]formamide (**42**) are formed. Two pathways are possible for the production of **42** (Scheme I): the preformed *N*,*N*-bis(hydroxymethyl)formamide (**40**) might be esterified by benzoyl chloride: alternatively, the intermediate monoester **41** could be hydroxymethylated by formaldehyde and subsequently benzoylated. Esterification of *N*-(hydroxymethyl)-*N*-methylformamide (**12**) proceeded straightforwardly to give **13** and **14**. The ether **10** was prepared from *N*-(hydroxymethyl)formamide (**9**) in acidic ethanol, the mechanism presumably involving trapping of the intermediate[17] *N*-formylmethyleneiminium ion (**43**) by ethanol (Scheme I).

The aminal **11** was synthesized by the Einhorn reaction[18] from dimethylamine, formaldehyde, and formamide. In this case, the iminium ion intermediate arises[19] from condensation of formaldehyde with the secondary amine rather than with formamide. The aminal was quaternized to afford the trimethylammonium iodide **44** in order to enhance the leaving-group ability of the amino group. A variety of basic conditions was examined in an effort to effect condensation of the salt with *N*-acetyl-L-cysteine: NMR analysis of the products confirmed the presence of *N*-acetyl-*S*-(formamidomethyl)cysteine (**45**), but a pure sample could not be isolated from the mixtures.

N,*N*-Dimethylglycine (**37**) was prepared in two efficient steps: substitution of bromide in methyl bromoacetate by dimethylamine was followed by acid hydrolysis of the ester to give the hydrochloride salt of **37** in 76% overall yield.

Synthesis of the "push–pull" olefins and vinylogous amides **29–35** involved condensation of the appropriate activated methyl and methylenic substrates with a dialkoxymethyl electrophile. Synthetic and conformational studies on nitro enamines **29–31** have been described by

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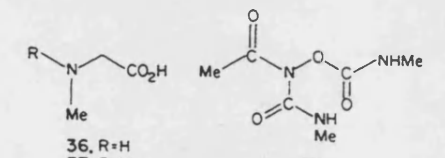
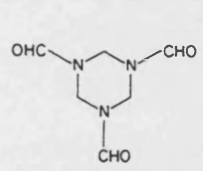


36, R=H
37, R=Me

38



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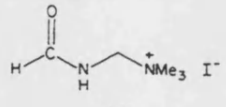

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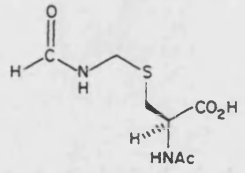

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us previously.[20] The morpholine analogue **32** was prepared in a three-component condensation (nitromethane, morpholine, and triethyl orthoformate). The ^{1}H NMR spectrum of this compound confirms *E* geometry about the C=C bond in solution in dimethyl sulfoxide ($J_{C1H,C2H}$ = 11 Hz); this coupling constant is diagnostic of trans coupling in **30** and **31**.[20] Treatment of preformed 2-cyano-3-ethoxypropenenitrile with methylamine in a conjugate addition–elimination reaction afforded the monomethyl dicyano enamine **33** whereas use of malononitrile as the activated methylenic reactant in condensation with dimethylformamide dimethyl acetal furnished the dimethyl analogue **34**. Attempts to prepare 2-nitro-3-(substituted amino)propenenitriles through condensation using the relatively unstable nitroacetonitrile (**46**) were unsuccessful.

The vinylogous amide **35** was formed in good yield from treatment of 3-methoxy-2-methylpropenal with methylamine again via a conjugate addition–elimination reaction. Proton NMR spectroscopy of **35** in chloroform showed the *E* conformation about the pro-amide C–N bond ($J_{C3H,NH}$ = 14 Hz). Also, compound **33** is shown to be a 5:1 mixture of rotamers in dimethyl sulfoxide with the *E* conformer ($J_{vinylCH,NH}$ = 15 Hz) about the pro-amide bond predominating over the *Z* form ($J_{vinylCH,NH}$ = 9 Hz).

Antitumor Activity

Of the range of amides screened against the TLX5 lymphoma (Table II), only NMF (**1**) (optimum T/C 191% at 800 mg kg^{-1}), its CD_3 analogue **3** (T/C 142% at 400 mg kg^{-1} day^{-1}), and the *N*-ethyl homologue **4** (T/C 137% at 800 mg kg^{-1} day^{-1}) display activity. The M5076 tumor was completely inhibited by NMF at a dose of 200 mg kg^{-1} day^{-1} for 17 days (Table III), but, of the other congeners of NMF tested, only formamide (**2**), *N*,*N*-dimethylformamide (DMF, **8**), *N*-(hydroxymethyl)formamide (**9**), and *N*-methylbenzamide (**21**) showed marginal activity. Particularly noteworthy is the inactivity of *N*-(2-chloroethyl)formamide (**5**) against the TLX5 lymphoma, which contrasts with the marked activity elicited by the 2-chloroethyl derivatives in the nitrosourea,[9] triazene,[10] and imidazotetrazine[11] series and suggests that the formamides may operate by a nonalkylating mechanism.

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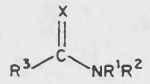
Table I



	R_1	R_2	R_3	X
1	Me	H	H	O
2	H	H	H	O
3	CD_3	H	H	O
4	Et	H	H	O
5	CH_2CH_2Cl	H	H	O
6	cyclopropyl	H	H	O
7	CH_2CF_3	H	H	O
8	Me	Me	H	O
9	CH_2OH	H	H	O
10	CH_2OEt	H	H	O
11	CH_2NMe_2	H	H	O
12	CH_2OH	Me	H	O
13	CH_2OAc	Me	H	O
14	CH_2O_2CPh	Me	H	O
15	Me	OH	H	O
16	Me	Me	H	S
17	Me	H	Me	O
18	Me	H	CF_3	O
19	Me	Me	Me	O
20	CH_2OH	H	CCl_3	O
21	Me	H	Ph	O
22	CH_2OH	H	Ph	O
23	OH	H	NH_2	O
24	Me	H	NHMe	O
25	Me	Me	NMe_2	O
26	Me	H	NHMe	S
27	Me	Me	NMe_2	S
28	Me	Me	NMe_2	NH
29	H	H	H	$CHNO_2$
30	Me	H	H	$CHNO_2$
31	Me	Me	H	$CHNO_2$
32	$-CH_2CH_2OCH_2CH_2-$		H	$CHNO_2$
33	Me	H	H	$C(CN)_2$
34	Me	Me	H	$C(CN)_2$
35	Me	H	H	C(Me)CHO

Many *N*-alkyl xenobiotics are metabolized to the corresponding α-hydroxy derivatives, some of which give rise to electrophiles.[16] Thus, the possibility[21] that NMF might require in vivo metabolic activation to *N*-(hydroxymethyl)formamide (**9**), or a conjugate thereof, was given initial credence when the carbinolamide was shown to possess potent inhibitory activity against the TLX5 lymphoma in vitro: however, this effect may have been mediated[21] by traces of cytotoxic formaldehyde that contaminated the sample of **9**. Attempts to purify the sample of **9** by fractional distillation led to fragmentation of the carbinolamide into starting materials, formaldehyde, and formamide. Four *N*-hydroxymethyl compounds **9** (a metabolite of NMF in mice),[22] **12** (the major metabolite of dimethylformamide in rodents),[23] and the carbinolamides **20** and **22**, derived from trichloroacetamide and benzamide, respectively, were all devoid of activity against the TLX5 lymphoma in vivo (Table II). Nevertheless, compound **9** showed marginal activity against the M5076 tumor (Table III), albeit at a very high dose (1600 mg kg^{-1} daily × 17). The disappointing in vivo results with these compounds may reflect an inherent lack of activity of *N*-hydroxymethyl amides per se, although *N*-hydroxymethyl compounds of the melamine[24] and triazene[10] series are at least

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Table II. Antitumor Activity of *N*-Methylformamide and Its Active Analogues[a] against the TLX5 Lymphoma[b] in Mice

no.	dose range tested, mg kg^{-1} day^{-1}	optimal dose,[c] mg kg^{-1} day^{-1}	mean death day[d] (±SD)	control death day (±SD)	T/C × 100%
1	800–100	800	17.6 (0.9)	9.2 (0.4)	191
		400	16.4 (1.3)	9.2 (0.4)	178
		200	12.4 (1.1)	9.2 (0.4)	135
		100	11.0 (0.7)	9.2 (0.4)	120
2	1200–50	800	11.2 (1.3)	9.1 (0.3)	123
3	800–50	400	18.8 (1.5)	13.2 (0.8)	142[e]
4	800–100	800	12.6 (1.3)	9.2 (0.4)	137
31	400–25	100	15.4 (1.3)	11.1 (0.3)	139

[a] Analogues 5, 7–15, 17, 20, 22, 23, 29, 30, 32–39 were found to be inactive against this tumor. The data for these compounds are presented in the supplementary material (see below). [b] The TLX5 lymphoma was passaged at 7-day intervals by ip injection of 2×10^5 cells into female CBA/ca mice (18–23 g). For antitumor tests 2×10^5 cells were injected subcutaneously into the inguinal region of female CBA/ca mice (18–23 g). Drugs were dissolved in physiological saline or Me_2SO/arachis oil (1:9) and administered daily for 5 days by ip injection commencing 72 h after tumor implantation. The mean day of death of groups of five animals were recorded and survival times of treated animals (T) compared with untreated controls (C). A value of T/C (×100%) of >125 is considered significant antitumor activity. [c] Except for compound 1 where a range of doses is described. [d] Value at optimal dose. [e] T/C × 100% for NMF (1) at this dose was 139 in this experiment.

Table III. Antitumor Activity of *N*-Methylformamide and Its Active Analogues[a] against the M5076 Reticulum Cell Sarcoma[b] in Mice

no.	dose range tested, mg kg^{-1} day^{-1}	LD_{10},[c] mg kg^{-1} day^{-1}	LD_{50},[c] mg kg^{-1} day^{-1}	optimal dose,[d] mg kg^{-1} day^{-1}	mean tumor volume (±SD)	control tumor volume (±SD)	T/C × 100%
1	400–6.25	220	300	200	NM	2.7 (0.5)	0
				100	0.3 (0.1)	2.7 (0.5)	11
				50	0.9 (0.2)	2.7 (0.5)	33
				25	2.1 (0.6)	2.7 (0.5)	70
2	400–100	200	270	200	0.8 (0.3)	2.1 (0.6)	38
8	1500–600	1130	1280	1000	1.0 (0.2)	2.5 (0.5)	40
9	2500–600	1580	1930	1500	0.8 (0.1)	2.1 (0.6)	38
21	800–100	450	650	400	0.8 (0.2)	1.9 (0.5)	42
31	200–25	55	75	50	0.5 (0.2)	1.5 (0.3)	35
38	150–6.25	53	63	50	0.9 (0.1)	2.4 (0.3)	38

[a] Analogues 4, 6, 12, 16–19, 24–28 were found to be inactive against this tumor. The data for these compounds are presented in the supplementary material (see below). [b] Fragments of tumor were obtained from donor BDF_1 mice (18–23 g). These were pooled, homogenized, and diluted with saline to produce a suspension of 10^7 cells mL^{-1}. Cells (10^6 in 0.1 mL) were injected intramuscularly into the left hind legs of groups of five female BDF_1 mice (18–23 g). Drugs were administered daily for 17 days by ip injection commencing 24 h after tumor implantation. Mean tumor volumes (in cm^3) were determined 24 days after tumor implantation. Tumor diameters were measured by calipers and the volumes calculated according to the following formula: volume = $(l \times w^2)/2$ where l is the longest tumor diameter and w the diameter perpendicular to the long axis. The volume of the leg without tumor is 0.1–0.2 cm^3 when measured in this manner. Tumors smaller than 0.2 cm^3 are "nonmeasurable" (NM). The mean tumor volumes of test animals (T) was compared to those of controls (C). A value of T/C × 100% of <42% is considered significant antitumor activity.[6] [c] Lethal dose values were estimated on day 24 from the number of survivors in the drug-treated groups. [d] Except for compound 1 where a range of doses is described.

as active as their *N*-methyl analogues. Alternatively, the inactivity of the *N*-hydroxymethyl derivatives in the formamide series may be due to pharmacokinetic problems associated with their high polarity. Successful chemical efforts to prepare lipophilic prodrug modifications of *N*-(hydroxymethyl)formamide (**9**) did not lead to biological activity. Thus, *N*-(ethoxymethyl)formamide (**10**), *N*-[(dimethylamino)methyl]formamide (**11**), and 1,3,5-triformylhexahydro-1,3,5-triazine (**39**) were all inactive in vivo against the TLX5 lymphoma as were the acetyl (**13**) and benzoyl (**14**) derivatives of *N*-(hydroxymethyl)-*N*-methylformamide (**12**).

Possibly, metabolic hydroxylation of NMF represents a bioinactivation as far as antitumor activity is concerned,[21] but its involvement in the hepatotoxicity of the drug cannot be excluded. Metabolic N-hydroxylation was also considered, but the possible product of such a pathway, *N*-hydroxy-*N*-methylformamide (**15**), a known teratogen,[25] proved inactive against the TLX5 lymphoma as did another *N*-hydroxy compound, the clinically used hydroxyurea **23**.

It has been shown recently[22] that the formyl carbon of NMF is extensively metabolized, being excreted by mice as carbon dioxide. The inactivity of a series of compounds where the formyl hydrogen is replaced by methyl or substituted methyl groups (**17–20**) or amino and substituted amino groups (**23–28**) and the corresponding inactivity of derivatives where the formyl oxygen is replaced by sulfur (**16, 26–27**) attests to the critical influence of the formyl group in mediating the activity of NMF. Compounds **21** and **38** do, however, possess marginal activity against the M5076 sarcoma. Replacement of the formyl group by substituted ethenyl residues to afford "push–pull" olefins **29–34** and the vinylogous NMF **35** led to loss of activity, except in the case of *N*,*N*-dimethyl-2-nitroethaneamine (**31**), which inhibited both tumors, although not markedly with T/C × 100% = 139 on TLX5 and T/C × 100% = 35 on M5076 experiments. Interestingly, the corresponding monomethyl compound **30**, arguably a closer analogue of NMF (with a 9:1 *Z*/*E* ratio of rotamers about the MeNH–C bond),[20] is inactive against the TLX5 lymphoma (Table II).

In conclusion, NMF (**1**) is the only compound in the series reported with good inhibitory activity against both the TLX5 lymphoma and M5076 reticulum cell sarcoma in vivo; any structural modification has a dyschemotherapeutic effect. These results are consistent with an earlier study[8] on the effects of formamides against the Ehrlich ascites tumor, which demonstrated that there is a rigorous structural requirement for antitumor activity. Other tumors do respond to certain of the agents described here: worthy of mention is the inhibitory activity of NMF (**1**),[26]

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N-(hydroxymethyl)formamide (9),[21] *N*-(acetoxymethyl)-*N*-methylformamide (13), and carecamide (38)[26] against the MX-1 mammary xenograft. Moreover, recent interest in NMF stems from the recognition that it is a prototype of a new class of low molecular weight antitumor agent capable of inducing terminal differentiation of human tumor cell lines such as the human promyelocytic leukemia HL60,[27] a property that it shares with a number of the congeners described in this paper. Results of these studies will be described in a future paper.[28]

Experimental Section

Unless otherwise stated, NMR spectra were obtained at 60 MHz with Varian EM360A and Varian A60A spectrometers. Other NMR spectra were acquired at 80 MHz and 220 MHz with Bruker WP80 and Perkin-Elmer R34 instruments, respectively. IR spectra were recorded with Pye Unicam SP200 and Perkin Elmer 1310 spectrometers. Melting points are uncorrected.

N-(Hydroxymethyl)-2,2,2-trichloroacetamide (20) was a kind gift of Professor Bundgaard (Copenhagen, Denmark) and *N,O*-bis-(*N*-methylcarbamoyl)acetohydroxamic acid (38, carecamide) was donated by the Drug Resources Branch of the National Cancer Institute. *N*-(Trideuteriomethyl)formamide (3),[13] *N*-(acetoxymethyl)-*N*-methylformamide (13),[15] *N*-(hydroxymethyl)benzamide (22),[15] 2-nitroethenamine (29),[20] *N*-methyl-2-nitroethenamine (30),[20] and *N,N*-dimethyl-2-nitroethenamine (31)[20] were prepared as previously described by us.

***N*-Ethylformamide (4).** Ethyl formate (74.0 g, 1.0 mol) was added to 70% aqueous ethylamine with cooling, and the mixture was stirred at ambient temperature for 2 h. Distillation gave *N*-ethylformamide (70.0 g, 96%) as a colorless liquid, bp 195–198 °C (lit.[29] bp 197–199 °C).

***N*-(2-Chloroethyl)formamide (5).** Sodium (2.30 g, 100 mmol) was dissolved in anhydrous ethanol (100 mL). Ethyl formate (25 mL), anhydrous Na_2CO_3 (20 g), and 2-chloroethylamine hydrochloride (8.15 g, 70 mmol) were added at −5 °C during 30 min. Stirring was continued at ambient temperature for 20 h before the mixture was filtered. Distillation of the filtrate afforded the formamide (5.50 g, 51%) as a colorless liquid: bp 98–100 °C (2 mm) (lit.[30] bp 79–95 °C (2 mm)); IR (liquid film) 3290, 1670, and 1520 cm^{-1}; NMR ($CDCl_3$) δ 3.5–3.7 (m, 4 H, CH_2CH_2), 7.5 (br, 1 H, NH), and 8.0–8.3 (m, 1 H, CHO); MS, *m/z* 109/107 (M^+).

***N*-Cyclopropylformamide (6).** Cyclopropylamine (11.4 g, 200 mmol) was added to a mixture of ethyl formate (50 mL), anhydrous Na_2CO_3 (30 g), and anhydrous ethanol (50 mL) at −5 °C, and the mixture was stirred at ambient temperature for 20 h. *N*-Cyclopropylformamide was isolated as for 5 above as a colorless liquid (10.0 g, 59%), bp 86–88 °C (3 mm); IR (liquid film) 3260 and 1670 cm^{-1}; NMR (220 MHz, $CDCl_3$) δ 0.9–1.1 (m, 4 H, CH_2CH_2), 2.6–2.85 (m, 1 H, cyclopropyl 1-H), 8.18 (s, 0.6 H, CHO of *Z* rotamer), and 8.29 (d, J = 12 Hz, 0.4 H, CHO (*E*)); MS, *m/z* 85 (M^+). Anal. (C_4H_7NO) C, H, N.

***N*-(2,2,2-Trifluoroethyl)formamide (7).** A mixture of sodium formate (680 mg, 10 mmol) and 2,2,2-trifluoroethylamine hydrochloride (1.35 g, 10 mmol) was heated gently under reflux for 20 min in the apparatus described previously[18] and then distilled. This product was redistilled (Kugelrohr) to give *N*-(2,2,2-trifluoroethyl)formamide (1.05 g, 83%) as a colorless liquid: bp 70–80 °C (3 mm); IR (liquid film) 3310, 1680, and 1540 cm^{-1}; NMR (220 MHz, $CDCl_3$) δ 3.96 (dq, J = 6.5 and 10 Hz, 1.8 H, CH_2CF_3 of *Z* rotamer), 8.11 (d, J = 11.5 Hz, 0.1 H, CHO (*E*)), 8.23 (s, 0.9 H, CHO (*Z*)), and 8.75 (br, 1 H, NH); MS, *m/z* 127 (M^+). Anal. ($C_3H_4F_3NO$) C, H, N.

***N*-(Ethoxymethyl)formamide (10).** A mixture of 40% aqueous KOH (2 mL, 14 mmol), formamide (45 g, 1 mol), and paraformaldehyde (33 g, 1.1 mol HCHO) was stirred for 30 min before ethanol (200 mL) and concentrated H_2SO_4 (3 mL) were added. The reaction mixture was stirred for a further 6 h and filtered. Anhydrous diethyl ether (25 mL) was added to the filtrate. The whole was allowed to stand over $NaHCO_3$ for 20 h, filtered, and concentrated under reduced pressure. The residue, in water (50 mL), was extracted with dichloromethane (5 × 100 mL). The combined extracts were washed with water and dried (Na_2SO_4) and the solvents evaporated under reduced pressure. Distillation gave *N*-(ethoxymethyl)formamide (22.0 g, 21%) as a colorless liquid: bp 108–120 °C (1 mm) (lit.[31] bp 71–82 °C (0.01–0.1 mm)); IR (liquid film) 3450 and 1670 cm^{-1}; NMR ($CDCl_3$) δ 1.16 (3 H, t, J = 7 Hz, CH_3), 3.53 (m, 2 H, CH_2CH_3), 4.7 (m, 2 H, NCH_2O), 7.1 (br, 1 H, NH), and 8.27 (m, 1 H, CHO); MS, *m/z* 103 (M^+).

***N*-(Dimethylaminomethyl)formamide (11).** Aqueous formaldehyde (37%, 112 g) was added dropwise to a mixture of formamide (45 g, 1 mol) and 40% aqueous dimethylamine (70 g) at 0 °C. The whole was stirred at ambient temperature for 20 h. Distillation furnished *N*-(dimethylaminomethyl)formamide (68.3 g, 67%) as a colorless oil: bp 100 °C (3 mm) (lit.[32] bp 62–65 °C (0.05 mm)); IR (liquid film) 3300 and 1650 cm^{-1}; NMR ($CDCl_3$) δ 2.25 (m, 6 H, $N(CH_3)_2$), 4.0 (m, 2 H, NCH_2N), 7.1 (br, 1 H, NH), and 8.2 (m, 1 H, CHO); MS, *m/z* 102 (M^+).

***N*-(Hydroxymethyl)-*N*-methylformamide (12)** was prepared by the method of Grady and Stott.[33] ^1H NMR showed the product to contain 5% *N*-methylformamide (1). The former decomposed on attempted distillation. *N*-(Hydroxymethyl)formamide (9) was similarly prepared.[33]

***N*-[(Benzoyloxy)methyl]-*N*-methylformamide (14).** *N*-(Hydroxymethyl)-*N*-methylformamide (11; 95%; 9.0 g, 96 mmol) and benzoyl chloride (11.5 mL) were stirred together in 5% aqueous NaOH (120 mL) at 0 °C for 30 min. The oily lower layer was washed with aqueous $NaHCO_3$. Distillation furnished the ester (5.2 g, 29%) as a colorless oil: bp 154 °C (3 mm) (lit.[34] bp 111–123 °C (0.02 mm)); IR (liquid film) 1720 and 1690 cm^{-1}; NMR (220 MHz; $(CD_3)_2SO$) δ 2.94 (s, 2.55 H, NCH_3 of *Z* rotamer), 3.14 (s, 0.45 H, NCH_3 (*E*)), 5.64 (s, 0.3 H, NCH_2 (*E*)), 5.72 (s, 1.7 H, NCH_2 (*Z*)), 7.66 (m, 3 H, Ar 3-, 4-, and 5-H), 8.08 (m, 2 H, Ar 2- and 6-H), 8.29 (s, 0.15 H, CHO (*E*)), and 8.70 (m, 0.85 H, CHO (*Z*)).

***N*-Hydroxy-*N*-methylformamide (15).** To *N*-methylhydroxylamine hydrochloride (835 mg, 10 mmol), in methanol (20 mL), was added sodium methoxide (540 mg, 10 mmol), followed by ethyl formate (20 mL). The whole was stirred for 16 h before being filtered through diatomaceous earth. The solvents were evaporated from the combined filtrate and methanol washings. Distillation of the residue gave the hydroxamic acid (610 mg, 81%) as a colorless oil: bp 140–147 °C (1 mm) (lit.[35] bp 60 °C (0.005 mm)); IR (liquid film) 2300 (br) and 1670 cm^{-1}; NMR ($CDCl_3$) δ 3.20 (br s, 3 H, CH_3), 7.8–8.2 (m, 1 H, CHO), and 9.73 (s, 1 H, OH).

***N*-Methyl-2,2,2-trifluoroacetamide (18).** Excess methylamine was passed through a solution of trifluoroacetic anhydride (12.6 g, 60 mmol) in diethyl ether (400 mL) during 2 h. The mixture was filtered and the filtrate was concentrated under reduced pressure. The residue was distilled at 3 torr to give *N*-methyl-2,2,2-trifluoroacetamide (4.44 g, 58%) as a white solid: mp 45 °C (lit.[36] bp 80 °C (3 mm), mp 48–49.5 °C); IR (Nujol mull) 3320, 1700, and 1560 cm^{-1}; NMR ($CDCl_3$) δ 2.95 (d, 3 H, J = 5 Hz, NCH_3) and 7.2 (br, 1 H, NH).

***N*-(2-Nitroethenyl)morpholine (32).** A mixture of trimethoxymethane (29.6 g, 279 mmol), morpholine (8.7 g, 100 mmol), nitromethane (30.5 g, 500 mmol), and toluene-4-sulfonic acid (500 mg) was boiled under reflux for 1 h. Evaporation of

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excess reagents under reduced pressure and passage of the residue through a short column of silica gel in CH_2Cl_2 followed by recrystallization from EtOH afforded *N*-(2-nitroethenyl)morpholine (10.4 g, 73%) as yellow needles: mp 142.5 °C (lit.[37] mp 140–141 °C); IR (KBr) 1625 cm^{-1}; NMR ($(CD_3)_2SO$) δ 3.63 (m, 8 H, CH_2), 7.00 (d, 1 H, J = 11 Hz, 2-H), and 8.30 (d, 1 H, J = 11 Hz, 1-H).

2-Cyano-3-(methylamino)propenenitrile (33). Excess methylamine was passed into a suspension of 2-cyano-3-ethoxypropenenitrile (1.22 g, 10 mmol) in diethyl ether (50 mL) at 0 °C and the whole was stirred at ambient temperature for 20 h. Evaporation of the solvent and excess methylamine under reduced pressure and recrystallization of the residue from anhydrous EtOH yielded 2-cyano-3-(methylamino)propenenitrile (770 mg, 72%) as orange microcrystals: mp 191–194 °C (lit.[38] mp 191–192 °C); IR (KBr) 3250, 2220, 2200, and 1640 cm^{-1}; NMR ($(CD_3)_2SO$) δ 2.97 (d, J = 4 Hz, 2.5 H, NCH_3 of *E* rotamer about vinyl–N bond), 3.07 (m, 0.5 H, NCH_3 (*Z*)), 7.53 (d, J = 9 Hz, 0.17 H, CH (*Z*)), 7.83 (d, J = 15 Hz, 0.83 H, CH (*E*)), and 8.8 (br, 1 H, NH); MS, m/z 107 (M^+).

2-Cyano-3-(dimethylamino)propenenitrile (34). Dimethylformamide dimethyl acetal (4.76 g, 40 mmol) and propanedinitrile (2.64 g, 40 mmol) were stirred together for 5 h and then concentrated under reduced pressure to give a yellow solid. Chromatography (silica gel, ethyl acetate) furnished the amino nitrile (1.0 g, 21%) as a white solid: mp 80.5–82 °C (lit.[39] mp 82–83 °C); IR (KBr) 2220, 2210, and 1650 cm^{-1}; NMR ($CDCl_3$) δ 3.23 (s, 3 H, NCH_3), 3.37 (s, 3 H, NCH_3), and 7.1 (s, 1 H, CH); MS, m/z 121 (M^+).

2-Methyl-3-(methylamino)propenal (35). Ethanolic methylamine (33%, 12.5 mL, 135 mmol) was added dropwise to 3-ethoxy-2-methylpropenal (11.4 g, 10 mmol) at 0 °C and the whole was stirred at ambient temperature for 20 h. Distillation gave a yellow oil (bp 114–118 °C (2 mm)), which was redistilled to afford 2-methyl-3-(methylamino)propenal (5.7 g, 58%): bp 114–116 °C (1 mm); mp 55 °C; IR 3260, 1660, and 1580 cm^{-1}; NMR (80 MHz, $CDCl_3$) δ 1.65 (s, 3 H, CCH_3), 3.07 (d, J = 3 Hz, 3 H, NCH_3), 6.54 (br, 1 H, NH), 6.86 (d, J = 14 Hz, 1 H, NCH), and 8.84 (s, 1 H, CHO); MS, m/z 99 (M^+). Anal. (C_5H_9NO) C, H, N.

***N*,*N*-Dimethylglycine (37).** Dimethylamine hydrochloride (50 g) was dissolved in H_2O (100 mL) and diethyl ether (400 mL) was added at 0 °C followed by sufficient anhydrous K_2CO_3 to dry the organic phase. Filtration gave an ethereal solution of dimethylamine to which was added methyl bromoacetate (15.3 g, 100 mmol), and the mixture was allowed to stand at ambient temperature for 20 h. The precipitated dimethylamine hydrobromide was removed by filtration and the filtrate was concentrated at atmospheric pressure. Distillation of the residue gave methyl (dimethylamino)acetate (9.13 g, 77%) as a colorless liquid: bp 45–51 °C (20 mm) (lit.[40] bp 50–54 °C (30 mm)); IR (liquid film) 1740 cm^{-1}. The above ester (9.13 g, 77 mmol) was boiled under reflux in 11 M hydrochloric acid for 24 h before evaporation of the excess reagent and drying at 10 torr in the presence of sodium hydroxide pellets gave *N*,*N*-dimethylglycine hydrochloride (10.70 g, 99%) as a white powder: mp 187–189 °C (lit.[41] mp 186–189 °C); IR (liquid film) 2930 (br) and 1740 cm^{-1}; NMR ($CDCl_3$) δ 2.9 (s, 6 H, $N(CH_3)_2$), 4.3 (s, 2 H, NCH_2), and 7.4 (br, 2 H, NH and OH).

1,3,5-Triformylhexahydro-1,3,5-triazine (39). Acetic anhydride (21.4 g) was added during 20 min to formic acid (9.7 g, 211 mmol) at −5 °C. Hexamethylenetetramine (6.7 g, 48 mmol) was added during 1.5 h such that the temperature remained below 10 °C, and then the mixture was stirred at ambient temperature for 2 h. Water (11 mL) was added and the mixture was neutralized by addition of K_2CO_3. After evaporation of the solvents under reduced pressure, the residue was extracted with $CHCl_3$. Drying (Na_2SO_4) and evaporation of the solvent furnished a white solid which was recrystallized from anhydrous EtOH to yield the hexahydrotriazine (2.4 g, 22%) as an hygroscopic white solid: mp 170.5–171.5 °C (lit.[42] mp 171–172 °C); IR 1710 and 1675 cm^{-1}; NMR ($CDCl_3$) δ 5.2 (s, 6 H, NCH_2N) and 8.25 (s, 3 H, CHO); NMR (CF_3CO_2H) δ 5.5 (s, 6 H, NCH_2N) and 8.46 (m, 3 H, CHO); MS, m/z 171 (M^+). Anal. ($C_6H_9N_3O_3$) C, H, N.

***N*-[(Benzoyloxy)methyl]formamide (41) and *N*,*N*-Bis[(benzoyloxy)methyl]formamide (42).** To *N*-(hydroxymethyl)formamide (4; 10.0 g, 133 mmol) and triethylamine (20 mL) in THF (100 mL) at 0 °C was added dropwise benzoyl chloride (17 mL). The mixture was stirred for 1 h at 0 °C, then filtered and concentrated under reduced pressure. The residue, in $CHCl_3$, was washed with $NaHCO_3$ and water and dried (Na_2SO_4), and the solvents were evaporated under reduced pressure. Chromatography (silica gel, chloroform) gave *N*-[(benzoyloxy)methyl]formamide (240 mg, 1%): mp 86–88 °C from aqueous MeOH; IR (KBr) 3250, 3150, 1710, and 1690 cm^{-1}; NMR (220 MHz, $CDCl_3$) δ 5.52 (d, J = 8.5 Hz, 0.67 H, CH_2 of *E* rotamer), 5.58 (d, J = 8.5 Hz, 1.33 H, CH_2 (*Z*)), 6.94 (br, 0.33 H, NH (*E*)), 7.07 (br, 0.67 H, NH (*Z*)), 7.52 (m, 2 H, Ar 3- and 5-H), 7.66 (m, 1 H, Ar 4-H), 8.11 (m, 2 H, Ar 2- and 6-H), 8.35 (s, 0.67 H, CHO (*Z*)), and 8.48 (0.33 H, d, J = 11 Hz, CHO (*E*)); MS, m/z 180 (M + 1). Anal. ($C_9H_9NO_2$) C, H, N. Evaporation of the solvents from earlier eluates afforded *N*,*N*-bis[(benzoyloxy)methyl]formamide (100 mg, 0.2%) as a colorless oil: IR (liquid film) 1720 and 1710 cm^{-1}; NMR ($CDCl_3$) δ 5.75 and 5.8 (2 x s, 4 H, CH_2), 7.3 (m, 6 H, 3-, 4-, and 5-H), 7.75 (m, 4 H, 2- and 6-H), 8.63 (s, 1 H, CHO). Anal. ($C_{17}H_{15}NO_5$) C, H, N.

***N*-(Formamidomethyl)trimethylammonium Iodide (44).** A mixture of iodomethane (10 mL) and *N*-[(dimethylamino)methyl]formamide (9; 5.1 g, 50 mmol) was stirred in diethyl ether at 0 °C for 2 h. The evaporation residue was recrystallized from EtOH to give *N*-(formamidomethyl)trimethylammonium iodide (5.9 g, 58%) as a white solid: mp 149–151 °C (lit.[43] mp 151–156 °C dec); IR (KBr) 3300 and 1695 cm^{-1}; NMR ($(CD_3)_2SO$) δ 3.1 (9 H, s, $N(CH_3)_3$), 4.75 (d, J = 7 Hz, 1.6 H, NCH_2N of *Z* rotamer), 5.00 (d, J = 7 Hz, 0.4 H, NCH_2N (*E*)), 8.45 (s, 0.8 H, CHO (*Z*)), 8.57 (m, 0.2 H, CHO (*E*)), and 9.4 (br, 1 H, NH).

Nitroacetonitrile (46). Freshly distilled thionyl chloride (4.3 mL) was added dropwise to a solution of 2-nitroacetaldoxime[44] (6.0 g, 58 mmol) in boiling anhydrous diethyl ether (40 mL) during 5 min, and the mixture was boiled under reflux for a further 1 h. Filtration and concentration under reduced pressure gave a yellow oil which, in diethyl ether, was washed with water and dried ($CaCl_2$), and the solvents were evaporated to give nitroacetonitrile (2.9 g, 58%) as a brown oil: IR (liquid film) 1560 and 1350 cm^{-1}; NMR ($CDCl_3$ + $(CD_3)_2SO$; 3:1) δ 5.85 (s, 2 H, CH_2); MS, m/z 120 (M^+).

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Registry No. 1, 123-39-7; 2, 75-12-7; 3, 87955-92-8; 4, 627-45-2; 5, 24589-68-2; 6, 58644-54-5; 7, 34005-37-3; 8, 68-12-2; 9, 13052-19-2; 10, 38952-30-6; 11, 28919-10-0; 12, 20546-32-1; 13, 1608-69-1; 14, 5129-78-2; 15, 21239-12-3; 16, 758-16-7; 17, 79-16-3; 18, 815-06-5; 19, 127-19-5; 20, 34891-76-4; 21, 613-93-4; 22, 6282-02-6; 23, 127-07-1; 24, 96-31-1; 25, 632-22-4; 26, 534-13-4; 27, 2782-91-4; 28, 80-70-6; 29, 29270-77-7; (*Z*)-30, 86602-47-3; (*E*)-30, 86602-50-8; (*E*)-31, 73430-27-0; (*E*)-32, 101419-83-4; 33, 79080-32-3; 34, 16849-88-0; 35, 101419-84-5; 36, 107-97-1; 37, 1118-68-9; 38, 81424-67-1; 39, 58793-59-2; 41, 101419-85-6; 42, 101419-86-7; 44, 52322-57-3; 46, 13218-13-8; HCO_2Et, 109-94-4; $Cl(CH_2)_2NH_2$·HCl, 870-24-6; HCO_2Na, 141-53-7; $F_3CCH_2NH_2$·HCl, 373-88-6; MeN-

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HOH·HCl, 4229-44-1; $(MeO)_3CH$, 149-73-5; O_2NCH_3, 75-52-5; $EtOCH{=}C(CN)_2$, 123-06-8; $Me_2NCH(OMe)_2$, 4637-24-5; $NCCH_2CN$, 109-77-3; $EtOCH{=}C(Me)CHO$, 42588-57-8; $BrCH_2CO_2Me$, 96-32-2; $Me_2NCH_2CO_2Me$, 7148-06-3; $Me_2NCH_2CO_2H$·HCl, 2491-06-7; $O_2NCH_2CH{=}NOH$, 5653-21-4; cyclopropylamine, 765-30-0; morpholine, 110-91-8.

Supplementary Material Available: Table IV, antitumor data for inactive analogues of *N*-methylformamide against the TLX5 lymphoma in mice. Table V, antitumor data for inactive analogues of *N*-methylformamide against the M5076 reticulum cell sarcoma in mice (2 pages). Ordering information is given on any current masthead page.

PUBLICATION 16

Selective Reactions in the Triazene Series. Part 2. Protodediazoniation of Arenediazonium Salts with Formamide

M. D. Threadgill and A. P. Gledhill

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Selective Reactions in the Triazene Series. Part 2.[1] Protodediazoniation of Arenediazonium Salts with Formamide

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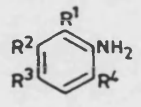
Treatment of preformed arenediazonium tetrafluoroborates or arenediazonium trifluoroacetates (formed *in situ*) with formamide and base effects reduction to the corresponding arene in moderate to good yield in cases where an electron-withdrawing substituent is present on the aromatic ring. Other functionalities remain unaffected. The mechanism of the protodediazoniation is shown to involve transfer of the formyl hydrogen atom to the substrate and may proceed *via* a 1-aryl-3-formyltriazene.

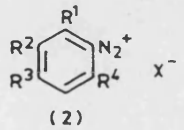
It has recently been shown that many *N*-methyl-containing compounds are metabolised by a preparation of murine liver to the corresponding *N*-hydroxymethyl derivatives[2] and, in the case of *N*-methylbenzamide, oxidised further to the N–CHO compound, *N*-formylbenzamide.[3] 1-Aryl-3,3-dimethyltriazenes are known antitumour agents but require prior oxidative metabolism for activity,[4] 3-(hydroxymethyl)triazenes being implicated as intermediates. In order to be able to test the hypothesis that these latter triazenes could be further metabolised, like the benzamides, to *N*-formyl analogues, a synthetic programme towards the preparation of 1-aryl-3-formyltriazenes was initiated. However, during this programme, the sole identifiable product (in moderate yield) from treatment of



(1)

a; $R^1 = R^2 = R^4 = H$, $R^3 = CO_2Me$
b; $R^1 = R^2 = R^4 = H$, $R^3 = CO_2Et$
c; $R^1 = R^2 = R^4 = H$, $R^3 = NO_2$
d; $R^1 = R^3 = H$, R^2 = 2,4-diamino-6-ethylpyrimidin-5-yl, $R^4 = Cl$
e; $R^1 = R^3 = H$, $R^2 = NO_2$, $R^4 = Me$
f; $R^1 = CN$, $R^2 = R^3 = R^4 = H$
g; $R^1 = OMe$, $R^2 = R^4 = H$, $R^3 = NO_2$
h; $R^1 = R^2 = R^4 = H$, $R^3 = Ac$
i; $R^1 = Ac$, $R^2 = R^3 = R^4 = H$
j; $R^1 = CO_2Me$, $R^2 = R^3 = R^4 = H$
k; $R^1 = Me$, $R^2 = R^3 = R^4 = H$
l; $R^1 = Me$, $R^2 = R^4 = H$, $R^3 = NO_2$
m; $R^1 = R^2 = R^4 = H$, $R^3 = OMe$
n; $R^1 = R^2 = R^4 = H$, $R^3 = Me$
o; $R^1 = R^2 = R^4 = Br$, $R^2 = H$
p; $R^1 = R^2 = R^4 = H$, $R^3 = Br$

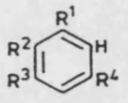
4-methoxycarbonylbenzenediazonium tetrafluoroborate (**2a**) with formamide and base was methyl benzoate (**3a**). Several agents have been reported to reduce arene diazonium salts to



(2)

R^1,R^2,R^3,R^4 as (1)
a—f; X = BF_4
g—p; X = CF_3CO_2

the corresponding arene, including tetrahydrofuran,[5] formaldehyde,[6] hypophosphorous acid,[7] *NN*-dimethylformamide[8] (DMF), and alcohols,[9] although problems have been encountered with each method. For example, biphenyls have been observed[9] as important by-products in the ethanol reduction. Formamide has once been claimed[8] to reduce a diazonium salt but the yield was reported to be markedly inferior to that achieved when the substrate was treated with DMF.



(3)

a; $R^1 = R^2 = R^4 = H$, $R^3 = CO_2Me$
b; $R^1 = R^2 = R^4 = H$, $R^3 = CO_2Et$
c; $R^1 = R^2 = R^4 = H$, $R^3 = NO_2$
d; $R^1 = R^3 = H$, R^2 = 2,4-diamino-6-ethylpyrimidin-5-yl, $R^4 = Cl$
e; $R^1 = R^3 = H$, $R^2 = NO_2$, $R^4 = Me$
f; $R^1 = CN$, $R^2 = R^3 = R^4 = H$
g; $R^1 = OMe$, $R^2 = R^4 = H$, $R^3 = NO_2$
h; $R^1 = R^2 = R^4 = H$, $R^3 = Ac$
i; $R^1 = Me$, $R^2 = R^4 = H$, $R^3 = NO_2$
j; $R^1 = R^3 = R^4 = Br$, $R^2 = H$
k; $R^1 = R^2 = R^4 = H$, $R^3 = Br$

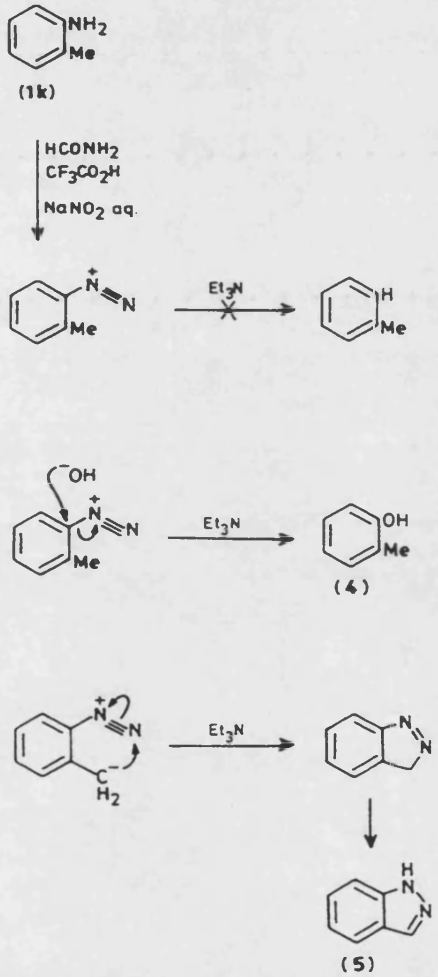
In order to investigate the generality of the reduction of (**1a**) by formamide, nitroaniline (**1c**) and 5-(3-amino-4-chlorophenyl)-6-ethylpyrimidine-2,4-diamine (**1d**) were diazotised in the usual way with sodium nitrite in aqueous tetrafluoroboric acid. The diazonium salts were isolated and dried before being dissolved in anhydrous formamide and treated with triethylamine. In both cases, good yields of the protodediazoniated product (**3c,d**) were obtained.

The method can be made into a true 'one-pot' synthetic reaction by carrying out the diazotisation with the formamide as solvent. Trifluoroacetic acid serves as the acid catalyst required. The intermediate diazonium trifluoroacetates were not isolated but the success of the sodium nitrite diazotisation step was indicated routinely by the formation of a bright orange-red azo-dye on treatment of a withdrawn aliquot with 2-naphthol and aqueous potassium carbonate. Addition at this stage of a large excess of triethylamine caused the rapid evolution of nitrogen gas characteristic of the reaction. Many arylamines were found to be deaminated by this facile process, as shown in the Table.

Substrates bearing only electron-donating substituents in the *para*-position, such as methoxy and methyl as in (**1m**) and (**1n**),

did not give the expected arene, only tarry materials resulting. However, where an electron-withdrawing substituent was present in substrate (**1g**) along with an electron-withdrawing moiety, protodediazoniation was achieved.

When 2-methylaniline (**1k**) was diazotised and treated with formamide and triethylamine according to the general one-pot procedure, the expected reduction product, toluene, was not observed by t.l.c. analysis of the reaction mixture. Here the isolated products were 2-methylphenol (**4**) and a trace of indazole (**5**). As with the diazonium salts (**2m,n**), 2-methylbenzenediazonium (**2k**) is insufficiently electrophilic to be reduced by the formamide but undergoes base-catalysed cyclisation to indazole (Scheme 1) and nucleophilic substitution by



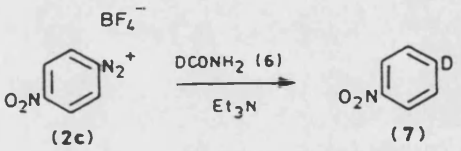
Scheme 1. Diazotisation of 2-methylaniline in formamide, followed by reaction with triethylamine gives products of substitution and cyclisation, not reduction

water to give the phenol. Indazole formation has been widely reported to be the outcome of attempted protodediazoniation of 2-methylbenzenediazonium salts; for example, the treatment of 2-methylanilines with pentyl nitrite and dioxane or tetrahydrofuran has been reported[5] to give indazoles as the sole products. When a powerful electron-withdrawing moiety, such as nitro, is present attached to the aromatic ring as in the cases of (**1e**) and (**1l**), we find indazole and phenol formation to be suppressed and good yields of the reduction products are obtained. This is consistent with Marx's observation[10] that 5-nitroindazole is not a product of the interaction of 2-methyl-4-nitrobenzenediazonium tetrafluoroborate with dimethylformamide.

Steric hindrance in the form of one *ortho*-substituent, as in the cases of the salts (**2b,e—g,i,j,l**) does not interfere with the progress of the reaction as shown by the moderate to good yields using these substrates or intermediates. However, where two bulky (bromo) substituents are present *ortho* to the diazonium function in salt (**2o**), the yield drops to 16%.

Mechanistic Studies.—The hydrogen atom which replaces the diazonium group on the arene could, at first sight, be derived from any of a number of sources including the formyl and NH_2 protons of the formamide, the triethylamine, or water. Water has been shown not to be the hydrogen source in a deuterium labelling study by Doyle *et al.*[8] in which 2,4,6-trichloroaniline was diazotised with t-butyl nitrite and then reduced by $HCONMe_2$ in the presence of one equivalent of D_2O. However, the tertiary amines tribenzylamine and *NN*-dimethylbenzylamine have been reported[11] to be the hydrogen source when 2,4,6-trichlorobenzenediazonium tetrafluoroborate was treated with the amines. In considering the formamide, Doyle *et al.*[8] also suggest that, since *NNN'N'*-tetramethylurea is as effective a reducing agent towards arenediazonium salts as is *NN*-dimethylformamide, then the formyl group is not the hydrogen source. This proposal is, however, weakened by their comparative study using *NN*-dimethylacetamide which was totally ineffective in reducing 4-nitrobenzenediazonium tetrafluoroborate (**2c**), whereas DMF gave a good yield of nitrobenzene.

In the present work, a sample of formamide deuteriated in the formyl group but not at the NH_2 function was prepared in a straightforward manner from 99 atom% methyl deuterioformate (DCO_2Me) and ammonia in anhydrous methanol. Insufficiently rigorous exclusion of moisture was found to lead to extensive hydrolysis of the starting ester. Treatment of the diazonium salt (**2c**) with this regiospecifically labelled formamide (**6**) and triethylamine furnished 4-deuterionitrobenzene (**7**) in which no pentaprotio compound could be detected by ^1H n.m.r. or mass spectroscopy. Hence, the formyl group is the sole hydrogen source under the conditions employed here (Scheme 2). This method using a relatively simply prepared isotopomer

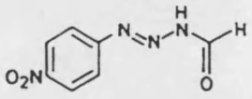


Scheme 2. Reaction of diazonium salt (**2c**) with deuterioformamide

of formamide may prove to be a useful method for the regiospecific labelling of aromatic compounds.

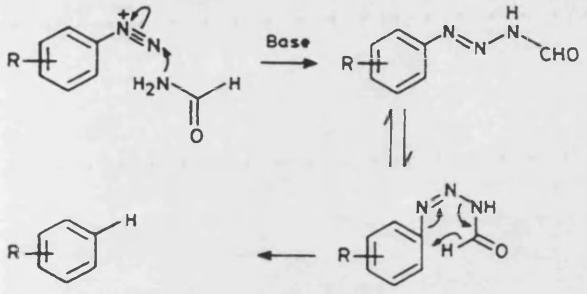
The question now arises as to whether the reduction process involves intermolecular hydride or $H^\bullet$ transfer from formamide to arene or, alternatively, is the originally sought 1-aryl-3-formyltriazene formed *in situ* as an intermediate followed by intramolecular hydride transfer (Scheme 3). In their comparative study of aryl diazocyanides and aryl diazoisocyanides, Ignasiak *et al.*[12] prepared 1-aryl-3-formyltriazenes in low yield by treatment of solid arene diazonium chlorides and formamide in ether with aqueous sodium hydrogen carbonate. In the present study, the tetrafluoroborate (**2c**) was subjected to similar treatment, giving, in low yield, 3-formyl-1-(4-nitrophenyl)triazene (**8**) accompanied by a quantity of the reduction

product, nitrobenzene (3c). Interestingly, Ignasiak reports the crude formyl triazenes to be oily solids; perhaps the liquid components were the corresponding reduction products. Reaction of (8) with triethylamine effected reduction smoothly to give (3c), thus implying that compound (8) is an intermediate



(8)

in the protodediazoniation reaction and that hydride transfer is intramolecular (Scheme 3). The absence of biphenyl derivatives from the products of these reactions is indicative that radical processes (*e.g.* H$^{\bullet}$ transfer) are not involved, unlike in cases where hypophosphorous acid [13] and alcohols [14] are the reductants.



Scheme 3. Proposed pathway of the reduction reaction

The identities of co-products arising from the reducing agent formamide have not been established. Consideration of the proposed reaction pathway would suggest that isocyanate ($^{-}$NCO) should be formed. However, attempts to trap it as 4-chlorophenylurea [15] by treatment of the mixture arising from (2c), formamide, and triethylamine with an excess of acetic acid and 4-chloroaniline were unsuccessful. Furthermore, the cyclic trimer, cyanuric acid (2,4,6-trihydroxy-1,3,5-triazine) was shown by t.l.c. to be absent.

In conclusion, a method for the efficient protodediazoniation of arenediazonium salts bearing electron-withdrawing substituents has been developed. The facility of this reaction may explain why *N*-formyltriazenes have not been reported as metabolites of the antitumour *N*-methyltriazenes in marked contrast to the situation in the benzamide series.[3]

Experimental

The following methods are illustrative. M.p.s are uncorrected. N.m.r. spectra were obtained at 60 MHz using a Varian EM360A spectrometer and a Perkin-Elmer 1310 instrument furnished the i.r. data.

Method A: Nitrobenzene (3c) *from 4-Nitrobenzenediazonium Tetrafluoroborate* (2c).—Triethylamine (375 mg, 3.7 mmol) was added to 4-nitrobenzenediazonium tetrafluoroborate (474 mg, 2.0 mmol) in formamide (2.0 ml). After the evolution of nitrogen had ceased (5 min), the red mixture was stirred at ambient temperature for a further 30 min. An ethyl acetate extract (20 ml) of the mixture was washed with water (4 × 25 ml), 2M-hydrochloric acid (2 × 20 ml), saturated aqueous sodium hydrogen carbonate (20 ml), and saturated brine (15 ml) before being dried (Na_2SO_4), filtered, and the solvent evaporated to give a dark oil. Preparative layer chromatography (silica gel, diethyl ether) furnished nitrobenzene (202 mg, 82%) as a very pale yellow oil.

Table. Methods and yields of protodediazoniation products

Substrate	Method	Product[a]	Yield (%)
(2a)	A	(3a)	58[b]
(2b)	A	(3b)	21[c]
(2c)	A	(3c)	82[b]
(2d)	A	(3d)	64[b]
(2e)	A	(3e)	78[c]
(2f)	A	(3f)	35[c]
(1g)	B	(3g)	67
(1h)	B	(3h)	25
(1i)	B	(3h)	29
(1k)	B	(3a)	73.5
(1j)	B	(4)[d]	20
		(5)[d]	Trace
(1l)	B	(3i)	53
(1m)	B		0
(1n)	B	[e]	0
(1o)	B	(3j)	16
(1p)	C	(3k)	60.5

[a] Products were characterised by n.m.r. and mass spectroscopy and had m.p.s or b.p.s consistent with reported values ('CRC Handbook of Chemistry and Physics', ed. R. C. Weast, CRC Press, Cleveland, Ohio, U.S.A., 1979, 58th edn.; P. B. Russell and G. H. Hitchings, *J. Am. Chem. Soc.*, 1951, 73, 3763; P. H. Griffiths, W. A. Walkey, and H. B. Watson, *J. Chem. Soc.*, 1934, 631). [b] Yield based on isolated arenediazonium tetrafluoroborate. [c] Yield based on starting aniline. [d] Toluene shown by t.l.c. to be absent. [e] Starting material (1n) (79%) recovered.

Method B: 3-Methoxynitrobenzene (3g) *from 2-Methoxy-4-nitroaniline* (1g).—Trifluoroacetic acid (2.0 g, 17.5 mmol) was added to 2-methoxy-4-nitroaniline (1.68 g, 10 mmol) in formamide (20 ml), followed by sodium nitrite (700 mg, 10 mmol) in water (1.3 ml) and the pale brown solution was stirred at ambient temperature for 20 min. Triethylamine (3.0 g, 30 mmol) was added and the deep red effervescing mixture was stirred for a further 20 min before being partitioned between ethyl acetate (100 ml) and water (50 ml). The organic portion was washed with 2M-hydrochloric acid (70 ml), water (4 × 50 ml), and saturated brine (50 ml), and then dried (Na_2SO_4) and filtered. The solvent was carefully evaporated to give a dark red oil which was chromatographed [silica gel, toluene–redistilled light petroleum (b.p. 60—80 °C); 1:1] to give 3-methoxynitrobenzene (1.03 g, 67%) as pale yellow crystals.

Method C: Bromobenzene (3k) *from Bromoaniline* (1p).—4-Bromaniline (1.72 g, 10 mmol) was treated as in Method B except that the crude product was distilled (Kugelrohr) to afford bromobenzene (950 mg, 60.5%) as a colourless liquid.

Deuterioformamide (6).—Anhydrous methanol (15 ml) (freshly distilled from magnesium methoxide) was saturated with ammonia at 0 °C. Methyl deuterioformate (99 atom %: Aldrich Chemical Co. Ltd.; 1.22 g, 20 mmol) was added and the mixture was stirred at ambient temperature in a tightly stoppered vessel for 16 h before careful evaporation of the solvent and excess ammonia gave deuterioformamide (890 mg, 97%) as a colourless liquid, b.p. 215 °C (lit.,[16] b.p. 111 °C at 20 mmHg for protio compound) $\nu_{max.}$ (liquid film) 3 300, 2 150 (C-D stretch), and 1 650 cm^{-1}; δ(D_2O) 5.2 (HOD); *m/z* 46 (M^{+}).

4-*Deuteronitrobenzene* (7).—4-Nitrobenzenediazonium tetrafluoroborate (2c) (237 mg, 1 mmol) was treated as for

Method A above except that deuterioformamide (6) (0.8 ml) and triethylamine (190 mg, 1.9 mmol) were employed. 4-Deuterionitrobenzene (96 mg, 77%) was obtained as a pale yellow liquid, $\nu_{max.}$ 2 950 (C-H stretch), 2 150 (C-D stretch), 1 520, and 1 330 cm^{-1}; $\delta(CDCl_3)$ 7.6 (2 H, *ca.* d, *J* 8 Hz, Ar 3- and 5-H), and 8.3 (2 H, d, *J* 8 Hz, Ar 2- and 6-H); *m/z* 124 (M^+).

3-Formyl-1-(4-nitrophenyl)triazene (**8**).—4-Nitrobenzenediazonium tetrafluoroborate (**2c**) (1.19 g, 5 mmol), formamide (900 mg, 20 mmol), water (10 ml), and diethyl ether (20 ml) were heated to reflux. Aqueous sodium hydrogen carbonate (8.4% w/v; 2.0 ml, 2 mmol) was added and the whole stirred under reflux for 15 min. The ether layer was removed and replaced by fresh ether (20 ml) and the $NaHCO_3$ addition/ether replacement cycle was repeated 5 times. The combined ethereal extracts were washed with water, dried, and the solvent evaporated to give an oily solid. This solid was triturated with light petroleum (b.p. 60—80 °C). The light petroleum supernatant was separated and the solvent was evaporated to give nitrobenzene (230 mg, 37%). The pale brown solid residue was characterised as 3-formyl-1-(4-nitrophenyl)triazene (280 mg, 29%) which decomposed when gently heated and failed to give a satisfactory CHN analysis although it appeared to be pure by t.l.c. (silica gel, toluene, and silica gel, light petroleum–dichloromethane; 2:1) $\nu_{max.}$(Nujol) 3 250, 1 710, 1 520, and 1 340 cm^{-1}; $\delta[(CD_3)_2SO]$ 6.2 (1 H, br, NH), 7.8 (2 H, *ca.* d, *J* 8 Hz) and 8.3 (2 H, d, *J* 8 Hz) (ArH), and 8.5 (1 H, br, CHO); *m/z* 194, 138, and 123.

Nitrobenzene (**3c**) *from 3-Formyl-1-(4-nitrophenyl)triazene* (**8**).—3-Formyl-1-(4-nitrophenyl)triazene (**8**) (194 mg, 1 mmol), was treated with triethylamine (2 ml) at ambient temperature for 4 h. Careful evaporation of the excess of reagent gave a dark residue which, on preparative layer chromatography (silica gel, diethyl ether) afforded nitrobenzene (81 mg, 66%) as a pale yellow oil.

Acknowledgements

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PUBLICATION 17

The Oxidative Decarboxylation of *N*-Aroylglycines to *N*-(Acetoxymethyl)benzamides and *N*-Formylbenzamides with Lead (IV) Acetate

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The Oxidative Decarboxylation of *N*-Aroylglycines to *N*-(Acetoxymethyl)benzamides and *N*-Formylbenzamides with Lead(IV) Acetate[1]

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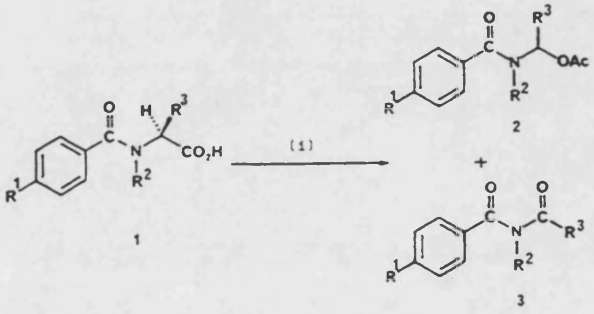
Treatment of *N*-aroylglycines that do not bear a strong electron-withdrawing substituent with lead(IV) acetate in acetic acid/acetic anhydride mixtures at 60–100 °C rapidly gives the corresponding *N*-(acetoxymethyl)benzamides and *N*-formylbenzamides in moderate yields after chromatography. These compounds are of interest in the study of the metabolism of xenobiotic *N*-methylbenzamides. *N*-(4-Nitrobenzoyl)glycine gives only *N*-(acetoxymethyl)-4-nitrobenzamide. 4-Chloro-*N*-methylbenzamide, *N*-aroylproline, and esters of *N*-aroylglycines are unaffected. Deuterium incorporation and other studies are consistent with a mechanism involving initial ligand exchange at lead followed by N-acetoxylation. Decarboxylation and elimination then ensue; final readdition of acetic acid to *N*-aroylimines leads to the observed products.

Many *N*-methyl-containing drugs and other compounds are metabolized to the corresponding *N*-hydroxymethyl analogue by preparations of murine liver.[2] The antitumor agent hexamethylmelamine,[3] the herbicide Monuron [*N*-(4-chlorophenyl)-*N'*,*N'*-dimethylurea],[4] and the common industrial solvent *N*,*N*-dimethylformamide[1] have been shown to be C-hydroxylated metabolically in vitro or in whole animals. The generated *N*-hydroxymethyl moieties are then either excreted as such or undergo further enzymic metabolism or intracellular chemical reaction. The relatively labile *N*-hydroxymethylamines may hydrolyze to give formaldehyde, a mutagen; while *N*-hydroxymethylbenzamide is sufficiently stable to be a substrate for further oxidation by cytosolic enzymes to *N*-formylbenzamide.[5] Hepatic metabolites containing the carbinolamine group may also act as electrophiles, either through the intermediacy of a small equilibrium concentration of the corresponding iminium ion or imine (recently reviewed[6]) or through biological derivatization of the alcohol (e.g., acetylation) which enhances its leaving group ability. We therefore sought a general preparation of *N*-(acetoxymethyl)benzamides and *N*-formylbenzamides as reference compounds for metabolic work and for study of their chemical reactivity in order to predict their biochemical reactions with cellular nucleophiles. Although *N*-(acetoxymethyl)benzamide (**2a**) has been prepared by acetylation of *N*-(hydroxymethyl)benzamide[7] and benzamide can be N-formylated with difficulty,[5] the present method furnishes both desired products from one reaction on one substrate. The oxidative decarboxylation of *N*-benzoylglycine (**1a**) by treatment with lead(IV) acetate to *N*-(acetoxymethyl)benzamide has been briefly reported by Süs and Rosenberger,[8] although the product was not well characterized. this paper now describes the results of our investigation into this reaction as regards synthetic utility, scope, limitations, and mechanistic pathway.

Scheme I. The Oxidative Decarboxylation of *N*-Aryl Amino Acids[a]



[a] (i) $Pb(OAc)_4/AcOH/Ac_2O$.

(1) Part 8 of the series "The Formation and Metabolism of *N*-Hydroxymethyl Compounds". For Part 7, see: Kestell, P.; Gill, M. H.; Threadgill, M. D.; Gescher, A.; Howarth, O. W.; Curzon, E. H. *Life Sci.* **1986**, *38*, 719.

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0022-3263/86/1951-3196$01.50/0

Table I

1	R^1	R^2	R^3	yield of 2, %	yield of 3, %
1a	H	H	H	10	22
1b	Cl	H	H	39[a]	28[a]
1c	Me	H	H	2.5[a]	11[a]
1d	NO_2	H	H	72[a]	0
1e	OMe	H	H	21[a]	12[a]
1f	Cl	Me	H	0	0
1g	Cl	H	Me	0	21[a]
1h	Cl	$CH_2CH_2CH_2$		0	0

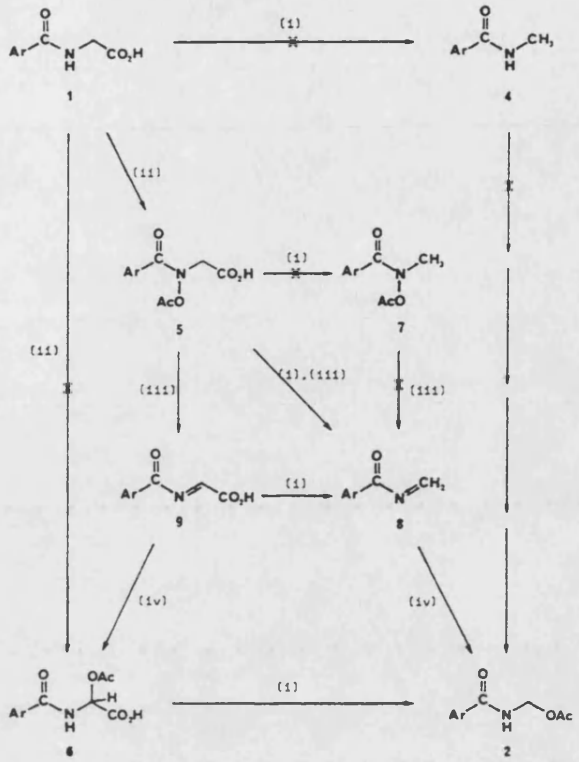
[a] Satisfactory analytical data (±0.4% for C, H, N) were reported for all new compounds listed in the table and for **2d**.

Aryl-substituted *N*-benzoylglycines **1a–c** and *N*-4-chlorobenzoyl amino acids **1f–h** were prepared by conventional Schotten–Baumann techniques[9] by addition of the aroyl chloride to the amino acid in aqueous sodium hydroxide solution at ambient temperature with vigorous stirring, acidification and collection of the *N*-aryl amino acid by filtration followed by recrystallization, usually from aqueous ethanol. *N*-(4-Chlorobenzoyl)sarcosine (**1b**) proved difficult to recrystallize but was purified after conversion to its diisopropylamine salt. The free acid was released by precipitation with hydrochloric acid from aqueous solution before oxidation was attempted.

N-(4-Chlorobenzoyl)glycine (**1b**) was the substrate used in preliminar experiments designed to optimize the reaction conditions so as to give acceptable yields of both products **2b** and **3b** in the same preparative run (Scheme I). The best yields were obtained by carrying out the reaction at 60 °C with reaction times between 30 min and 2 h in a solvent mixture comprising ca. 10% acetic anhydride and 90% acetic acid and by use of between 1 and 2 equiv of lead(IV) acetate. Higher temperatures, such as the 100 °C employed by Sūs[8] or boiling under reflux, speeded the already conveniently rapid reaction but also facilitated side reactions; significantly lower yields of both products were isolated. The reaction did not proceed at a measurable rate at ambient temperature. As shown in Table I, substitution at various positions in the substrate hippuric acids has a profound effect on the outcome of the reaction. Substitution in the para position of the benzoyl moiety with groups that are not strongly electron-withdrawing as in **1a–c,e** gave moderate to good yields of both oxidized products **2a–c,e** and **3a–c,e**. However, when a strong electron-withdrawing group is present on the aromatic ring, as in the nitro group of substrate **1d**, only the acetoxymethyl compound **2d** could be isolated. Interestingly, the maximum yield (72%) of **2d** was obtained using 2 equiv of lead(IV) acetate. Hence, it can be seen that the product distribution is markedly affected by the electronic nature of the para substituent; further oxidation taking place when a methoxy group (Hammett substituent constant $\sigma = -0.27$)[10] is present on the aromatic ring but not with the nitro derivative ($\sigma = +0.78$).[10]

Substitution at nitrogen, in the case of substrate **1f**, leads to a reaction mixture in which neither *N*-(acetoxymethyl)-4-chloro-*N*-methylbenzamide (**2f**) nor 4-chloro-*N*-formyl-*N*-methylbenzamide (**3f**) could be identified. TLC analysis showed that **1f** was very largely unaffected by the oxidant. The presence of a methyl group at the α-carbon of the glycine moiety (in substrate **1g**), on the other hand, did not prevent oxidative decarboxylation. In this case, the sole isolated material was the doubly oxidized product *N*-acetyl-4-chlorobenzamide (**3g**) in modest yield.

Scheme II. Possible Mechanistic Pathways for the Oxidative Decarboxylation of Hippuric Acids 1 to *N*-(Acetoxymethyl)benzamides 2[a–c]



[a] Paths with ⊣ have been shown by experiment not to take place. [b] (i)–CO_2; (ii) $Pb(OAc)_4$; (ii2) –AcOH; (iv) +AcOH. [c] Ar = 4-$R^1C_6H_4$ (R^1 as in Table I).

It may be that the 1-acetoxy ethyl compound **2g** was formed during the reaction but was unstable. No oxidized products were obtained from **1h** which has substitution at both nitrogen and aliphatic carbon by annulation.

An attempt to extend the scope of the synthetic reaction by selection of a different lead(IV) carboxylate was unsuccessful. Neither of the expected oxidative decarboxylation products [4-chloro-*N*-(trifluoroacetoxymethyl)-benzamide and **3b**] were obtained from treatment of substrate **1b** with a solution of lead(IV) oxide in a mixture of trifluoroacetic acid and trifluoroacetic anhydride. The former ester may be hydrolyzed during the mildly basic aqueous workup, but the corresponding alcohol, 4-chloro-*N*-hydroxymethylbenzamide, was also not obtained as a product of the reaction.

Mechanistic Studies. As shown in Scheme II, the first intermediates on the path from starting hippuric acid **1** to the first isolated oxidation product **2** may arise either from initial decarboxylation (in the case of the *N*-methylbenzamide **4**) or from acetoxylation (to give **5** or **6**). However, the intermediacy of **4** can be discounted on the following grounds. First, no trace of the corresponding *N*-methylbenzamide **4** is evident on TLC examination (compared with authentic materials[5,11]) of any of the reaction mixtures involving lead(IV) acetate oxidative of hippuric acids **1**. Second, although there are several reports[12] of oxidation of *N*-alkyl compounds α to nitrogen

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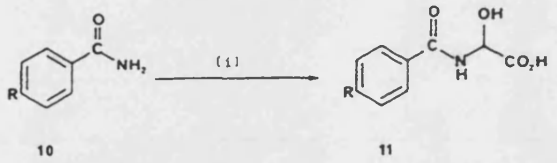
(10) Ritchie, C. D.; Sager, W. F. *Prog. Phys. Org. Chem.* **1964**, *2*, 323.
(11) Auerbach, J.; Zamore, McF.; Weinreb, S. M. *J. Org. Chem.* **1976**, *41*, 725.
(12) Galliani, G.; Rindone, B.; Scolastico, C. *Tetrahedron Lett.* **1975**, 1285.

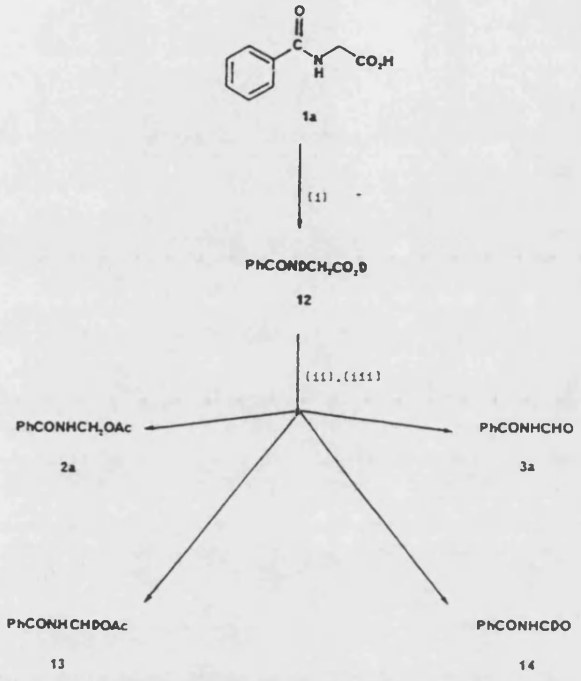
Scheme III. Condensation of 4-Chlorobenzamide with Glyoxylic Acid[a]



10 11

[a] (i) $OHCCO_2H$/1,4-dioxane.

Scheme IV. Deuterium Incorporation Experiments[a]

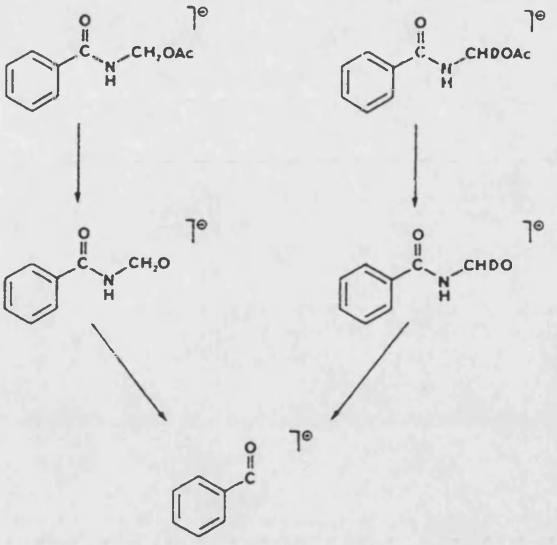


[a] (i) D_2O; (ii) $Pb(OAc)_4/CH_3CO_2D/Ac_2O$; (iii) $H_2O/NaHCO_3$.

by acetates of metals in high oxidation states, no consumption of 4-chloro-*N*-methylbenzamide (**4b**) was observed under the general conditions employed. Hence oxidation precedes rather than succeeds decarboxylation.

Several experiments were conducted to investigate whether oxidation occurs at carbon, converting **1** directly to the 2-acetoxyhippuric acid **6**, or at nitrogen, giving the *N*-acetoxyhippuric acid **5**. In the first, *N*-(4-chlorobenzoyl)-2-hydroxyglycine **11** was prepared in excellent yield by treatment of 4-chlorobenzamide **10** with glyoxylic acid hydrate (Scheme III). All attempts to acetylate **11** to give the proposed intermediate 2-acetoxy-*N*-(4-chlorobenzoyl)glycine (**6b**) by using acetic anhydride or acetyl chloride in the presence of inorganic or organic base were unsuccessful, intractable mixtures being obtained. With **6b** thus not available as an isolated characterizable material for further investigation, a more indirect approach was sought. Esterification of **11** to give **6b** might well take place in the warm acetic acid/acetic anhydride medium of the principal oxidative decarboxylation experiments. Treatment of **11** with such Ac_2O/AcOH mixtures in the presence or absence of lead(II) acetate (added to mimic the main reaction conditions more closely) gave the acetoxymethyl compound **2b**. Replacement of the nonoxidizing lead(II) acetate by lead(IV) acetate led to markedly decreased yields of **2b**. Hence, it is highly likely, but not completely rigorously proven, that at least some of the yield of **2** in the oxidation of **1** with lead(IV) acetate

Scheme V. Partial Mass Fragmentation of 2a



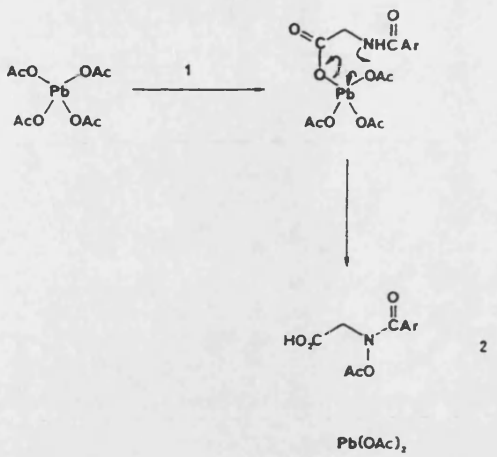
Scheme VI. Proposed Simultaneous Elimination/Decarboxylation

5

arises through the intermediacy of **6**.

The most useful evidence for contributions (or otherwise) of pathways to the overall reaction sequence comes from the deuterium incorporation experiments shown in Scheme IV. For ease of interpretation of the mass spectroscopic analysis of products, the unsubstituted benzoyl series (**1a**, etc.) was employed for these experiments. The carboxylic acid and amine NH protons of hippuric acid (**1a**) were exchanged for deuterium to an extent greater than 95% (judged by ^1H NMR in $CDCl_3$) by repeated recrystallization from deuterium oxide. The oxidative decarboxylation was then carried out under the standard conditions using lead(IV) acetate (free from acetic acid), monodeuterioacetic acid (CH_3CO_2D; 98 atom %), and acetic anhydride [$(CH_3CO)_2O$], followed by normal aqueous workup. Of the *N*-(acetoxymethyl)benzamide formed, 60–80% (varying between experiments) was found by mass spectrometry not to contain deuterium (*m*/*z* 193, 150), i.e., to be $PhCONHCH_2OAc$. A corresponding 20–40% of the material contained one deuterium atom ($PhCONHCHDOAc$). Examination of the mass spectral fragmentation pattern confirmed the location of the deuteron in the methylene group (Scheme V). As expected, no ions corresponding to the dideuterio compound, $PhCONHCD_2OAc$, were observed, and control experiments showed that deuterium is neither gained nor lost by exchange of hydrogens of product **2a** with solvent. These data are consistent only with the initial formation of the *N*-acetoxyhippuric acid **5**, followed by a mixed mechanism. The major proportion of **5** then undergoes a simultaneous elimination/decarboxylation, giving **8** (Scheme VI). With the minor part, elimination of acetic acid gives the imine carboxylic acid **9**, which could then either add acetic acid (giving **6**) or decarboxylate (giving **8**). Evidence for the involvement of **6** is presented below in that it appears to

Scheme VII. A Possible Mechanism of N-Acetoxylation of Hippuric Acids by Lead(IV) Acetate



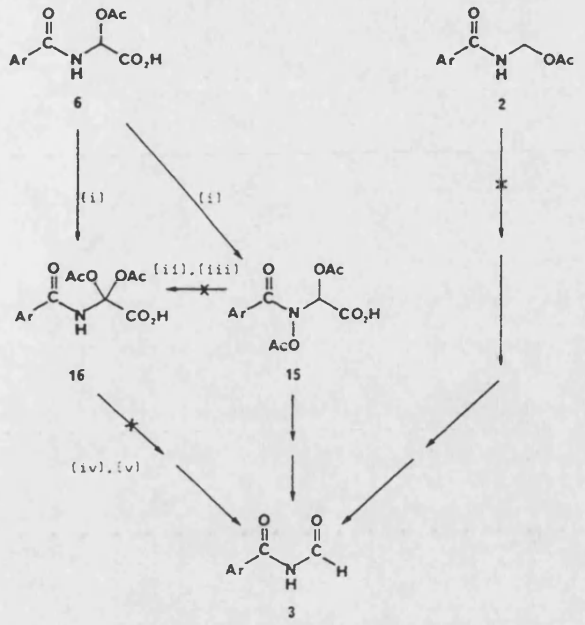
be the intermediate which is further oxidized, giving **3** as the final product.

A related reaction has been reported[13] in triazene chemistry in that 3-hydroxy-3-methyl-1-phenyltriazene has been converted to 3-(acetoxymethyl)-3-acetyl-1-phenyltriazene by treatment with acetic anhydride via elimination/addition. However, no *N*-(acetoxymethyl)-4-chlorobenzamide (**2b**) was formed when 4-chloro-*N*-hydroxy-*N*-methylbenzamide was heated with mixtures of acetic acid and acetic anhydride in the presence or absence of lead(II) acetate, thus ruling out **7** as an intermediate. This observation is consistent with our previous report that this *N*-methylhydroxamic acid does not eliminate water to generate an iminium ion on treatment with trifluoroacetic acid.[14] As shown in Table I, *N*-(4-chlorobenzoyl)glycine (**1b**) is readily converted to acetoxymethyl compound **2b**, whereas all attempts to carry out the same conversion on the *N*-blocked analogue, *N*-(4-chlorobenzoyl)-*N*-methylglycine (**1f**), to give **2f** failed. Thus, direct C-acetoxylation of **1** to **6** does not occur. Additionally, none of the expected further oxidation product, **3f**, could be isolated from this reaction. Hence, it would appear that the NH is important for the reaction to take place; a deduction that supports the formation of the *C*-acetoxy compound **6** via an elimination/addition rearrangement from the *N*-acetoxy species **5**.

It would appear that the carboxylic acid moiety is essential for the reaction to take place since no oxidation of methyl [(4-chlorobenzoyl)amino]acetate could be detected under the usual reaction conditions. A rationalization could be that the initial step of the N-acetoxylation is carboxylate "ligand" exchange at lead (Scheme VII). This would be followed by an essentially intramolecular oxidation at nitrogen.

The reaction pathways leading to the further oxidation products **3** are much more complex, involving steps of oxidation, elimination, addition, and, perhaps, decarboxylation, culminating in hydrolysis on workup. The first question (Scheme VIII) is whether the isolable product of the first oxidation, **2**, is itself acetoxylated or whether one of the other intermediates in Scheme II is the substrate for lead(IV) acetate, leading ultimately to the *N*-formylbenzamide **3**. A pure sample of **2b** was subjected to

Scheme VIII. Possible Pathways to *N*-Formylbenzamides[a,b]



[a] Paths with ~~X~~ have been shown by experiment not to take place. [b] (i) $Pb(OAc)_4$; (ii) −AcOH; (iii) +AcOH; (iv) $-CO_2$; (v) hydrolytic workup.

treatment with lead(IV) acetate under the standard conditions. No conversion to **3b** was observed, and the sole effect of higher temperatures and/or longer reaction times led to decomposition of the starting *N*-(acetoxymethyl)-4-chlorobenzamide to unidentifiable products. This result shows that compounds **2** are indeed subject to oxidation by lead(IV) acetate but that this oxidation is degradative. Thus **2** does not lie on the pathway from **1** to **3**. This finding is further supported by the total lack of deuterium incorporation into product **3a** when the overall reaction is carried out in $Pb(OAc)_4/CH_3CO_2D/Ac_2O$ mixture.

The involvement of **6** in the formation of **3** is also indicated by the successful experimental conversion of the glyoxylic acid adduct **11** to **3b** under the standard experimental conditions. One may therefore speculate that the *C*-acetoxy compound **6** is the intermediate actually oxidized. As in the first stage oxidation of **1** above, acetoxylation either at carbon or at nitrogen is conceivable. However, the *C*,*C*-diacetoxy compound **16** (Scheme VII) is not an intermediate since decarboxylation of **16** in the deuterate medium would lead, after subsequent hydrolysis, to PhCONHCDO rather than the sole oberved moiety PhCONHCHO (**3a**). Clearly, intermediates of type **16** are impossible in the observed conversion of **1g** to **3g**. The excellent yield (72%) of **2d** under the standard conditions together with the complete absence of the *N*-formyl compound **3d** in the *p*-nitro series also lends weight to the proposal that **6** is directly N-acetoxylated to give **15**. The nitro group would have the effect of markedly reducing the electron density at the amide nitrogen atom, thus disfavoring oxidation by an electrophilic oxidant. It is tempting to speculate that a simultaneous elimination and oxidation step is involved, which is similar to that shown in Scheme VI for the other series of products.

In conclusion, it can be seen that a useful simultaneous synthetic route to the *N*-(acetoxymethyl)benzamides **2** and *N*-formylbenzamides **3** has been developed. These compounds are likely to be of biological and toxicological interest as weak electrophiles. The characterization of their

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(14) Addison, S. J.; Cunningham, B. D. M.; Gate, E. N.; Shah, P. Z.; Threadgill, M. D. *J. Chem. Soc., Perkin Trans. 1* 1985, 75.

chemical and biochemical electrophilic reactivity is in progress and will be presented in a future paper.[15] The mechanism of the oxidative decarboxylation of hippuric acids has been shown to involve two simultaneous routes to the first isolated oxidation products 2, but only one intermediate is further oxidized leading to compounds 3.

Experimental Section

General. IR spectra were determined as Nujol mulls. NMR spectra were obtained at 60 MHz with a Varian EM 360A spectrometer using tetramethylsilane as internal standard. Mass spectrometric studies were carried out with a VG Micromass 12 instrument in electron-impact mode at 70 eV. Melting points are uncorrected.

***N*-(4-Chlorobenzoyl)-*N*-methylglycine Diisopropylamine Salt (1f).** A mixture of 4-chlorobenzoyl chloride (8.75 g; 50 mmol), *N*-methylglycine (4.45; 50 mmol), KOH (5.6 g, 100 mmol), and water (100 mL) was stirred at ambient temperature for 3 h before being acidified by addition of 10 M hydrochloric acid. The resulting suspension was extracted with CH_2Cl_2. This extract was dried (Na_2SO_4) and filtered and the solvent evaporated under reduced pressure to give the acylsarcosine 1f as a slightly gummy solid, mp 119–121 °C (lit.[16] mp 133–134 °C). The melting point could not be raised by recrystallization from aqueous ethanol. The solid, in ethanol (100 mL), was treated with diisopropylamine (20 mL), and the volatile materials were again evaporated. Recrystallization of the residue from propan-2-ol gave the diisopropylamine salt of *N*-(4-chlorobenzoyl)-*N*-methylglycine (10.9 g; 66%) as white needles: mp 129–130 °C; IR 2700, 2450, 1630, 1560 cm^{-1}; NMR ($CDCl_3$) δ 1.30 (12 H, d, J = 7 Hz, $(CH_3)_2CH$), 3.10 (3 H, s, NCH_3), 3.27 (2 H, septet, J = 7 Hz, $(CH_3)_2CH$), 3.75 (2 H, s, NCH_2), 7.2–7.6 (6 H, m, Ar H, N^+H_2). Anal. Calcd for $C_{16}H_{25}ClN_2O_3$: C, 58.45; H, 7.65; N, 8.5. Found: C, 58.15; H, 7.65; N, 8.35.

***N*-(4-Chlorobenzoyl)-L-alanine (1g).** 4-Chlorobenzoyl chloride (5.25 g, 30 mmol) was added to L-alanine (2.67 g; 30 mmol) and KOH (3.9 g, 70 mmol) in water (50 mL). After being stirred vigorously for 13 h, the mixture was acidified by addition of 10 M hydrochloric acid and extracted with ethyl acetate. The extract was washed with saturated aqueous NaCl and dried (Na_2SO_4) and the solvent evaporated under reduced pressure to afford, after recrystallization from aqueous propan-2-ol, the *N*-acylalanine 1g (5.05 g, 74%) as white needles: mp 161–163 °C; IR 3290, 2750, 1710, 1640, 1600, 1550 cm^{-1}; NMR (2:1 $CDCl_3/(CD_3)_2SO$) δ 1.45 (3 H, d, J = 7 Hz, $CHCH_3$), 4.52 (1 H, quintet, J = 7 Hz, alanine H), 5.4 (1 H, br, CO_2H), 7.43 (2 H, d, J = 8 Hz) and 7.92 (2 H, d, J = 8 Hz) [Ar H], 8.45 (1 H, d, J = 7 Hz, NH). Anal. Calcd for $C_{10}H_{10}ClNO_3$: C, 52.75; H, 4.45; N, 6.15. Found: C, 52.5; H, 4.35; N, 6.05.

***N*-(Acetoxymethyl)-4-chlorobenzamide (2b) and 4-Chloro-*N*-formylbenzamide (3b).** *N*-(4-Chlorobenzoyl)glycine (1b) (6.27 g, 30 mmol) and $Pb(OAc)_4$ (13.29 g, 30 mmol) were stirred at 60 °C for 10 min in acetic acid (80 mL) and acetic anhydride (8 mL) before evaporation of the solvents under reduced pressure. The residue, in CH_2Cl_2, was washed twice with water, twice with saturated aqueous $NaHCO_3$, and once with saturated aqueous NaCl before being dried (Na_2SO_4) and filtered. Evaporation of the solvent under reduced pressure gave a viscous yellow oil, which, on chromatography [silica gel; 1:4 ethyl acetate–redistilled light petroleum (bp 60–80 °C)], furnished, as the faster running fraction, 4-chloro-*N*-formylbenzamide (3b) (1.53 g, 28%) as a pale yellow solid: mp 95–96 °C; IR 3280, 1730, 1685 cm^{-1}; NMR ($CDCl_3$) δ 2.00 (3 H, s, Ac), 5.40 (2 H, d, J = 7 Hz, NCH_2O), 7.40 (2 H, d, J = 9 Hz) and 7.85 (2 H, d, J = 9 Hz) [Ar H], 8.25 (1 H, br t, J = 7 Hz, NH); mass spectrum m/z 185/183 (M^+). The slower fraction, after evaporation of the eluant under reduced pressure, comprised *N*-(acetoxymethyl)-4-chlorobenzamide (2b) (2.69 g, 39%) as an off-white powder: mp 163–165 °C; IR 3320, 1730, 1650 cm^{-1}; NMR ($CDCl_3$) δ 7.55 (2 H, d, J = 8 Hz) and 8.10 (2 H, d, J = 8 Hz) [Ar H], 9.50 (1 H, d, J = 10 Hz, CHO), 10.7 (1 H, br, NH); mass spectrum, m/z 229/227 (M^+).

***N*-(Acetoxymethyl)benzamide (2a) and *N*-Formylbenzamide (3a).** *N*-Benzoylglycine (1a) (4.5 g, 25 mmol) was treated with $Pb(OAc)_4$ (20.5 g, 50 mmol), and the products were isolated as for the preparation of 2b and 3b above. *N*-Formylbenzamide (3a) (80 mg, 22%) was obtained as a pale yellow solid: mp 109–110 °C (lit.[17] mp 112–113 °C); IR 3280, 1730, 1685 cm^{-1}; NMR ($CDCl_3$) δ 7.5–8.3 (5 H, m, Ar H), 9.45 (1 H, d, J = 10 Hz, CHO), 10.5 (1 H, br, NH); mass spectrum, m/z 193 (M^+), 150, 122, 121, 105 (100). *N*-(Acetoxymethyl)benzamide (2a) (480 mg, 10%) was isolated as a colorless viscous oil identical with a sample previously prepared by us by another route.[7]

***N*-(Acetoxymethyl)-4-methylbenzamide (2c) and *N*-Formyl-4-methylbenzamide (3c).** *N*-(4-Methylbenzoyl)glycine (1c) (9.65 g, 50 mmol) was treated with $Pb(OAc)_4$ (41.0 g, 92.5 mmol), and the products were isolated as for the reaction of 1b above. *N*-Formyl-4-methylbenzamide (3c) (920 mg; 11%) was obtained as a white solid: mp 104 °C; IR 3320, 1745, 1690 cm^{-1}; NMR ($CDCl_3$) δ 2.40 (3 H, s, $ArCH_3$), 7.25 (2 H, d, J = 8 Hz) and 7.90 (2 H, d, J = 8 Hz) [ArH], 9.35 (1 H, d, J = 9 Hz, CHO), 10.45 (1 H, ca. d, J = 9 Hz, NH); mass spectrum, m/z 163 (M^+), 135, 119 (100), 91. *N*-(acetoxymethyl)-4-methylbenzamide (2c) (260 mg, 2.5%) was obtained as a white solid: mp 49 °C; IR 3320, 1760, 1670 cm^{-1}; NMR ($CDCl_3$) δ 2.00 (3 H, s, $COCH_3$), 2.35 (3 H, s, $ArCH_3$), 5.40 (2 H, d, J = 7 Hz, NCH_2O), 7.15 (2 H, d, J = 8 Hz) and 7.75 (2 H, d, J = 8 Hz) [Ar H], 8.30 (1 H, t, J = 7 Hz, NH); mass spectrum, m/z 207 (M^+), 164, 148, 119 (100).

***N*-(Acetoxymethyl)-4-nitrobenzamide (2d).** *N*-(4-Nitrobenzoyl)glycine (1d) (5.60 g, 25 mmol) was treated with $Pb(OAc)_4$ (22.15 g, 50 mmol) as for the preparation of 2b above except that chromatography was replaced by recrystallization from ethyl acetate/light petroleum (bp 60–80 °C). The acetoxymethylbenzamide 2d (4.28 g, 72%) was obtained as a very pale greenish yellow solid: mp 94 °C (lit.[18] mp 120 °C); IR 3370, 1760, 1670 cm^{-1}; NMR ($CDCl_3$) δ 2.10 (3 H, s, $COCH_3$), 5.45 (2 H, d, J = 7 Hz, NCH_2O), 7.90 (1 H, ca. t, J = 7 Hz, NH), 8.05 (2 H, d, J = 8 Hz) and 8.35 (2 H, d, J = 8 Hz) [Ar H].

***N*-(Acetoxymethyl)-4-methoxybenzamide (2e) and *N*-Formyl-4-methoxybenzamide (3e).** *N*-(4-Methoxybenzoyl)-glycine (1e) (2.99 g, 10 mmol) was treated with $Pb(OAc)_4$ (8.86g; 29 mmol) as for the preparation of 2b and 3b above. *N*-Formyl-4-methoxybenzamide (3e) (215 mg, 12%) was obtained as a white solid: mp 42–46 °C dec; NMR ($CDCl_3$) δ 3.60 (3 H, s, OCH_3), 6.95 (2 H, d, J = 8 Hz) and 7.85 (2 H, d, J = 8 Hz) [Ar H], 9.30 (1 H, d, J = 9 Hz, CHO), 10.0 (1 H, br, NH); mass spectrum, m/z 179 (M^+). *N*-(Acetoxymethyl)-4-methoxybenzamide (2e) (470 mg, 21%) was a white waxy solid of indefinite low mp: NMR ($CDCl_3$) δ 2.00 (3 H, s, $COCH_3$), 3.55 (3 H, s, OCH_3), 5.35 (2 H, d, J = 7 Hz, NCH_2O), 7.00 (2 H, d, J = 8 Hz) and 7.75 (2 H, d, J = 8 Hz) [Ar H], 8.60 (1 H, br, NH); mass spectrum, m/z 223 (M^+).

***N*-Acetyl-4-chlorobenzamide (3g).** *N*-(4-Chlorobenzoyl)-L-alanine (1g) (910 mg, 4 mmol) was treated with $Pb(OAc)_4$ (2.22 g, 5 mmol) as for the preparation of 2b and 3b above. *N*-Acetyl-4-chlorobenzamide (3g) (166 mg, 21%) was obtained as a colorless oil: IR 3370, 1700, 1650 cm^{-1}; NMR ($CDCl_3$) δ 2.60 (3 H, s, $COCH_3$), 7.50 (2 H, d, J = 8 Hz) and 7.80 (2 H, d, J = 8 Hz) [Ar H], 9.6 (1 H, br, NH); mass spectrum m/z 199/197 (M^+).

***N*-(4-Chlorobenzoyl)-2-hydroxyglycine (11).** 4-Chlorobenzamide (3.3 g; 20 mmol) was stirred with glyoxylic acid hydrate (1.84 g; 20 mmol) in 1,4-dioxane (50 mL) at 60 °C for 1 h. Evaporation of the solvent under reduced pressure afforded the adduct 11 (4.1 g; 89%) as white crystals: mp 150 °C dec; IR 3300, 2750, 1720, 1665 cm^{-1}; NMR (1:1 $CDCl_3/(CD_3)_2SO$) δ 5.75 (1 H, d, J = 8 Hz, NCHO), 7.50 (2 H, d, J = 8 Hz) and 7.90 (2 H, d, J = 8 Hz) [Ar H], 8.0 (1 H, br, CO_2H), 8.20 (1 H, brs, CHO*H*), 8.95 (1H, d, J = 8 Hz, NH).

Reaction of *N*-(4-Chlorobenzoyl)-2-hydroxyglycine (1f) with Acetic Acid and Acetic Anhydride. A solution of 11 (500 mg, 2.2 mmol) in acetic acid (4.0 mL) and acetic anhydride (2.0 mL) was stirred at 60 °C for 4 h in the presence of $Pb(OAc)_2$ (800 mg, 2 mmol). Evaporation of the solvents under reduced pressure gave a pale yellow gum, which, on column chromatography [silica

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(17) Thompson, Q. E. *J. Am. Chem. Soc.* 1951, 73, 5914.
(18) Zawadowska, I.; Zawadowski, T. *Acta Pol. Pharm.* 1969, 26, 409.

gel; 1:4 ethyl acetate–redistilled light petroleum (bp 60–80 °C)], afforded *N*-(acetoxymethyl)-4-chlorobenzamide (**2b**) (80 mg; 12%) with physical properties identical with those of the sample described above.

Reaction of *N*-(4-Chlorobenzoyl)-2-hydroxyglycine (11) with Lead(IV) Acetate. Glyoxylic acid adduct 11 (500 mg, 2.2 mmol) was treated with $Pb(OAc)_4$ (1.8 g, 4.06 mmol) according to the general method for the preparation of **2b** and **3b** above to furnish 4-chloro-*N*-formylbenzamide (**3b**) (110 g, 27%) with physical properties identical with those of the sample described above.

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PUBLICATION 18

S-(*N*-Methylcarbamoyl)-*N*-acetylcysteine; a Urinary Metabolite of the Hepatotoxic Experimental Antitumour Agent *N*-Methylformamide in Mouse, Rat and Man

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RAPID COMMUNICATION

S-(N-METHYLCARBAMOYL)-N-ACETYLCYSTEINE: A URINARY METABOLITE OF THE HEPATOTOXIC EXPERIMENTAL ANTITUMOUR AGENT N-METHYLFORMAMIDE (NSC 3051) IN MOUSE, RAT AND MAN.

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N-Methylformamide (NMF, NSC 3051) is an antineoplastic agent in mice[1,2]. In clinical trials in which the potential of NMF for the therapy of human cancers was evaluated, manifestations of liver damage were observed[3,4,5]. The mouse appears to be particularly sensitive to the hepatotoxic properties of NMF[6,7] and results of mechanistic studies in mice suggest that a reactive metabolite of NMF is responsible for its hepatotoxicity[7,8]. Whereas NMF is metabolised *in vitro* only to a very minor extent by liver fractions or isolated mouse hepatocytes, it undergoes extensive metabolism *in vivo* in rodents[9]. Carbon dioxide, methylamine and N-hydroxymethylformamide have been identified as major metabolites of NMF[10]. In that study, a further metabolite was detected but not characterised. We now report the identification of a new urinary metabolite of NMF and suggest that its precursor(s) may well be responsible for the hepatotoxicity and/or the antitumour activity of NMF.

MATERIAL AND METHODS

Chemicals. The labelled isotopomers of NMF, N-methyl-[^{14}C]formamide and N-[^{14}C]-methylformamide, were prepared as previously described[11]. S-(N-Methylcarbamoyl)-N-acetylcysteine was prepared as follows: Methyl isocyanate (2.0 g, 35 mmol) was added to N-acetyl-L-cysteine (4.1 g, 25 mmol) in anhydrous pyridine at 0°C and the mixture was stirred at this temperature for 4 days with the exclusion of moisture. The solvent was evaporated under reduced pressure. The residue was dissolved in dichloromethane and extracted with water. The aqueous extract was finally freeze-dried to furnish S-(N-methylcarbamoyl)-N-acetylcysteine (3.7 g, 67%) as a very hygroscopic white solid, MPt 65°C.

Drug administration and metabolite isolation and identification. Details of the i.p. administration of NMF or [^{14}C]-NMF (five daily doses of 400 mg/kg) to male CBA/CA mice (18-25 g) and male Wistar rats (300-350 g) and of the subsequent urine collection have been described previously[10,12]. Human urine samples were obtained from seven patients who received NMF (i.v. or p.o., dose range 300 mg/m^2 to 1200 mg/m^2) as a single dose. Urine samples were collected every 6 hr during a 24 hr period after drug administration. Details of the patients' age, sex and malignancies and of the formulation of NMF have been reported previously[4]. Urine samples were frozen after collection and pooled prior to analysis. After addition of 1M HCl to achieve pH1, samples were freeze-dried. The residues were suspended in methanol and subjected to TLC analysis using silica gel 60 coated plates (0.2 mm or 2 mm thickness, Merck AG, Darmstadt, W. Germany). Plates were developed in butan-1-ol:water:methanol (8:2:1 v/v). Thioesters were detected by spraying the plates either with 1M aqueous NaOH followed by Ellman's reagent[13] or with chloroplatinate reagent[14]. [^{14}C]-Labelled metabolites were detected as described by Kestell *et al.*[10]. On preparative TLC, the area of silica at the Rf value of the material which gave a reaction with Ellman's reagent after alkaline hydrolysis was removed from the plate and eluted with methanol. The solvent was evaporated at room temperature. The light brown residue was freeze-dried to remove traces of butan-1-ol. The metabolite isolated as described above or authentic S-(N-methylcarbamoyl)-N-acetylcysteine were dissolved in methanol (5 ml). HCl gas was bubbled through the solutions for 30 min. The solutions were left standing overnight at room

temperature, after which the solvent was evaporated under reduced pressure. The material was purified by HPLC with methanol:water (3:1 v/v) as eluant, and a FRAC-100 fraction collector (LKB, Croydon, U.K.). The eluate was concentrated under reduced pressure at 40°C. HPLC analysis was performed using a Waters trimodular system (Waters Associates, Northwich, U.K.), a Waters RCM-100 radial compression unit and a C_{18} 5 μm reverse-phase column. Radioactivity in the column eluate was detected by an HPLC ESI-Panax 506C radioactivity monitor (Rotherol and Mitchell Ltd., Ruislip, U.K.); u.v. detection was achieved with a Waters λ_{max} 480 LC spectrophotometer set at 205 nm. The eluant (either 0.01M octylamine in water adjusted to pH6 with concentrated HCl:methanol, 3:1 v/v; or water:methanol, 3:1 v/v) was pumped through the column at a flow rate of 1 ml/min. ^{1}H-NMR spectra were obtained at 400 MHz using a Bruker WH400 spectrometer with D_2O or borate buffer pH8/D_2O as solvents. Mass spectra were determined on a VG 7070 mass spectrometer in the chemical ionization mode, using 2-methylpropane as reagent gas. Spectra were run at a scan rate of 1 sec/decade and were processed on a VG 2035 data system.

RESULTS

TLC analysis of samples obtained by freeze-drying pooled urine of mice or rats which had received NMF or $^{[14]}$C-NMF or of patients who had been treated with NMF afforded a chromatographic band (Rf 0.30) which gave a positive reaction with the chloroplatinate reagent and with Ellman's reagent subsequent to alkaline hydrolysis. This material was absent in control urine samples. On HPLC analysis the material extracted from the TLC plate afforded one predominant peak detected by either its uv absorbance or monitoring of its radioactive label. The retention time of the metabolite was indistinguishable from that of authentic S-(N-methylcarbamoyl)-N-acetylcysteine.

The 400 MHz ^{1}H-NMR spectra of S-(N-methylcarbamoyl)-N-acetylcysteine at pH8 and of the metabolite isolated from patients' urine are shown in Fig. 1. In the spectrum of the reference compound the protons of the acetyl group resonate as a 3H singlet at δ2.01 ppm and the N-methyl moiety gives rise to a 3H singlet at δ2.78 ppm. The remaining protons of the molecule, the cysteine α-CH and β-CH_2, form an ABX system. The magnetic inequivalence of the β-methylene protons arises from the prochirality of the CH_2 in the presence of a chiral centre. The α-H appears as δ4.40 ppm (1H, dd, J 7.6 Hz and 4.2 Hz), β-H as δ3.16 ppm (1H, dd, J 14.3 Hz and 7.6 Hz), β'-H as δ3.43 ppm (1H, dd, J 14.3 Hz and 4.2 Hz). The geminal coupling constant of 14.3Hz and the two vicinal coupling constants (7.6 Hz and 4.2 Hz) are similar to the corresponding J values previously reported by us for other mercapturic acids[15]. At this alkaline pH, slow degradation to unidentified products was observed. In the ^{1}H-NMR spectrum of the metabolite (Fig. 1), the general pattern is very similar and the coupling constants are identical to those of the synthetic material.

Both isolated metabolite and authentic reference compound were treated with methanolic HCl and thus converted to a derivative which migrated to Rf 0.70 on TLC analysis. The chemical ionisation mass spectra of the esterified products are shown in Fig. 2 and show excellent congruity. Each spectrum has principal ions at m/z 235, 178, 136 and 60 which are due to (M+H) ions of the complete methyl ester molecule, N-acetylcysteine methyl ester (loss of MeNCO), cysteine methyl ester (subsequent loss of ketene) and acetamide respectively. Although the spectra presented in Figs. 1 and 2 were obtained with samples isolated from patients' urine, the material isolated from the urine of mice and rats afforded spectra almost identical to those shown in the figures.

DISCUSSION

Studies of the mechanism by which NMF causes hepatotoxicity in mice have accrued strong evidence for the contention that it is metabolised to a reactive species responsible for toxicity[2,7,8,9,16]. However the identification of carbon dioxide, methylamine and the pharmacologically innocuous N-(hydroxymethyl)formamide[17] as metabolites of NMF *in vivo*[10] gives but little indication as to the nature of this species. NMF itself is not hydrolysed to methylamine and formic acid by liver fractions *in vitro*. One might speculate that products of oxidation of NMF such as N-methylcarbamic acid (HOCONHMe) or the potent toxin

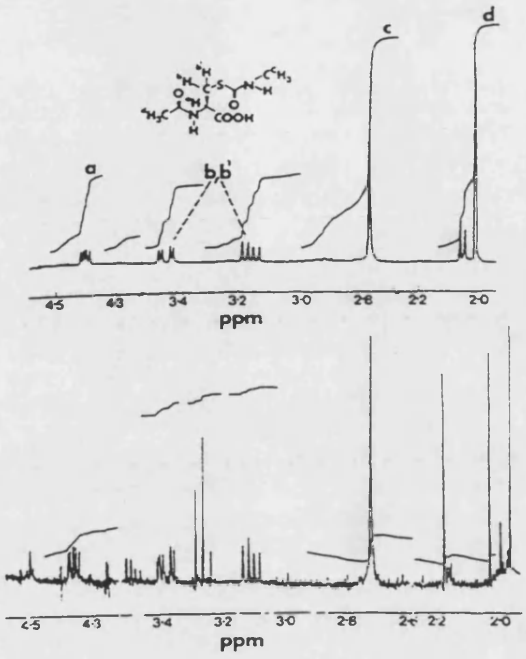

Fig. 1 Salient features of the ^{1}H-NMR spectra with D_2O of S-(N-methylcarbamoyl)-N-acetyl-cysteine (top panel) and the metabolite which was isolated by preparative TLC from the urine of patients who received NMF (bottom panel). The presence of alkaline buffer in the solution of authentic sample caused some degradation as shown by the signals near δ2.92, 3.23, 4.28 and 4.48 ppm. The chemical shifts of the synthetic mercapturic acid were found to be markedly sensitive to changes in pH in aqueous solution. It may be that the borate buffer used to mimic the pH of the solution of the isolated metabolite was not of precisely the same pH or ionic strength thus causing the small discrepancies in chemical shifts. The ^{1}H-NMR spectrum of an extract of control urine obtained by preparative TLC in the same way in which the metabolite was isolated showed resonances at δ2.17, 3.24, 3.27 and 3.29 ppm which can also be seen in the lower spectrum.

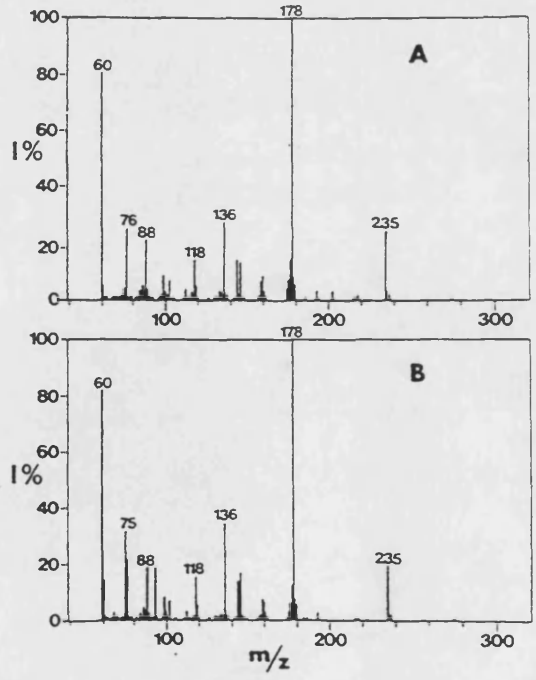

Fig. 2 Mass spectra of the methyl esters of (A) authentic S-(N-methylcarbamoyl)N-acetyl cysteine and (B) the metabolite isolated from the urine of patients who had received NMF.

methyl isocyanate (MeNCO) are the immediate precursors of the observed methylamine. Alternatively, these or other oxidation products of NMF would be able to form conjugates. In this report, the identification of such a conjugate, S-(N-methylcarbamoyl)-N-acetyl-cysteine, as a metabolite of NMF in the urine of mice, rats and men is described. Hydrolysis of N-methylthiocarbamates, such as this metabolite, indeed leads to methylamine and carbon dioxide. It is highly likely that the mercapturic acid characterised in this study results from metabolic modification of the corresponding S-(N-methylcarbamoyl)glutathione generated in the liver. The formation of this latter compound from NMF formally involves an oxidation at the formyl carbon of NMF, but the mechanistic route for this novel metabolic step is yet an enigma. It is noteworthy that N-alkylmonothiocarbamate compounds are electrophiles and carbamoylating agents and S-(N-alkylcarbamoyl)cysteines, compounds structurally related to the novel metabolite of NMF described here, have been reported to be potently cytotoxic[18].

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PUBLICATION 19

The Formation and Metabolism of *N*-Hydroxymethyl Compounds. IX. *N*-(Acetoxymethyl)-4-chlorobenzamide, an Electrophile but not a Mutagen in *Salmonella Typhimurium*

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THE FORMATION AND METABOLISM OF *N*-HYDROXYMETHYL COMPOUNDS—IX

N-(ACETOXYMETHYL)-4-CHLOROBENZAMIDE: AN ELECTROPHILE BUT NOT A MUTAGEN IN *SALMONELLA TYPHIMURIUM*

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Abstract—The electrophilic properties of 4-chloro-*N*-(hydroxymethyl)benzamide as a model compound of carbinolamides formed during the metabolic oxidation of *N*-methylamides were investigated. 4-Chloro-*N*-(hydroxymethyl)benzamide did not react with nucleophiles such as cyanide or glutathione under physiological conditions. In contrast, *N*-(acetoxymethyl)-4-chlorobenzamide yielded the cyanomethylamide with KCN and *S*-(4-chlorobenzamidomethyl)glutathione with glutathione. Under non-aqueous conditions, *N*-(acetoxymethyl)-4-chlorobenzamide reacted avidly with ethanethiol, with methanol and with diethylamine in the presence of base, whereas 4-chloro-*N*-(hydroxymethyl)benzamide did not afford products under these conditions. These results show clearly that *N*-(acetoxymethyl)-4-chlorobenzamide is the precursor of reactive electrophilic methyleneimines. 4-Chloro-*N*-(hydroxymethyl)benzamide was not biotransformed to such electrophilic species when incubated with mouse hepatic microsomes or a microsomal supernatant with acetyl-CoA or a PAPS generating system. Neither 4-chloro-*N*-(hydroxymethyl)benzamide nor its acetate ester were mutagenic in the short term bacterial assay using *Salmonella typhimurium*. Nevertheless, esters of carbinolamides, such as *N*-(acetoxymethyl)-4-chlorobenzamide, might possess toxic or carcinogenic properties.

Compounds having *N*-methyl moieties are prone to oxidative metabolism to the corresponding *N*-hydroxymethyl analogues. Frequently, the latter compounds decompose to formaldehyde and the N–H derivative. However, in some xenobiotics, low electron density at nitrogen confers chemical stability on these *N*-hydroxymethyl metabolites [2]. Thus, for example, the *N*-hydroxymethyl metabolites of *N*-methylamides can either be excreted unchanged or undergo further oxidative metabolism [3].

We have recently reviewed the evidence which shows that metabolically generated carbinolamines and carbinolamides can be the precursors of electrophilic methyleneiminium or methyleneimine species, metabolites which may be toxins [4]. The formation of these species might be the consequence of the loss of hydroxide or the elimination of water, respectively. Alternatively, iminium ions or imines derived from *N*-methyl compounds may be the products of metabolically generated esters of the *N*-hydroxymethyl compounds rather than the *N*-hydroxymethyl compounds themselves [4]. In this report, work is described in which these hypotheses have been investigated. 4-Chloro-*N*-(hydroxymethyl)benzamide and its acetate ester, *N*-(acetoxymethyl)-4-chlorobenzamide, were synthesised as model compounds from which formaldehyde is not readily released [3]. In addition to a comparison of their ability to form reactive species, their genotoxic potential was investigated as measured by the *Salmonella* mutation assay. In this study, the acetate ester of 4-chloro-*N*-(hydroxymethyl)benzamide (a possible albeit unlikely metabolite) was chosen as a model ester for the sulphate analogue which is a difficult synthetic target. The results of these investigations help to explain certain toxic properties of *N*-methyl compounds such as the anticancer drug hexamethylmelamine [5], the herbicide Monuron [6] and some *N*-methylaniline derivatives [7, 8] which require metabolism to exert their toxicity and which are known or strongly suspected to undergo metabolism to their *N*-hydroxymethyl derivatives.

MATERIALS AND METHODS

Chemicals. The following substances were prepared generally according to published methods: 4-chloro-*N*-(hydroxymethyl)benzamide [2, 9], *N*-(acetoxymethyl)-4-chlorobenzamide [1], 4-chloro-*N*-(cyanomethyl)benzamide [10] and *S*-(4-chlorobenzamidomethyl)glutathione [11]. The latter compound is novel and was characterised by NMR spectroscopy: δ [400 MHz; $(CD_3)_2SO$] 2.1 (2H, m, glutamyl β-CH_2), 2.35 (2H, m, glutamyl γ-CH_2), 2.72 (1H, dd, J 13.5 and 10.0 Hz) and 3.07 (1H, dd, J 13.5 and 4.4 Hz) (cysteine β-CH_2), 3.77 (2H, d, J

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For Part VIII of this series, see ref. [1].

BP 35:23-D

5.9 Hz, glycine CH_2), 3.9 (1H, m, glutamyl α-CH), 4.38 (1H, dd, J 13.7 and 2.2 Hz) and 4.48 (1H, dd, J 13.7 and 4.0 Hz) (NCH_2S), 4.61 (1H, m, cysteine α-H), 7.55 (2H, d, J 8.6 Hz) and 7.91 (2H, d, J 8.6 Hz) (ArH), 8.7 (7H, br, NH and OH) and 9.37 (1H, t, J *ca* 6 Hz, glycine NH).

$K^{14}CN$ was purchased from Amersham International (Amersham, U.K., specific activity: 59 mCi/mmol). The following materials were obtained from Sigma Chemical Co. (Poole, U.K.): acetyl-CoA, ATP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP. The benzamide derivatives were added to the incubation mixtures dissolved in DMSO or acetonitrile (25 μl).

Animals. Male BALB/c mice (19–24 g) were used for all metabolism experiments. In the bacterial mutagenicity tests, 9000 *g* supernatant fractions of livers from male Wistar rats (approximately 250 g) which had received Aroclor were used.

Studies of chemical reactivity. To investigate the reactivity of *N*-(acetoxymethyl)-4-chlorobenzamide with nucleophiles, the following chemical experiments were conducted. *N*-(Acetoxymethyl)-4-chlorobenzamide (341 mg, 1.5 mmol) was dissolved in methanol (5 ml). TLC analysis (silica gel, diethyl ether) showed no change after 15 min at ambient temperature or 5 min at 64°. Triethylamine (202 mg, 2 mmol) was added to the cooled mixture whereupon TLC showed quantitative conversion. Dichloromethane (20 ml) was added and the mixture was washed with aqueous toluene-4-sulphonic acid, water and saturated brine before being dried (Na_2SO_4). The solvents were evaporated under reduced pressure to give *4-chloro-N-(methoxymethyl)benzamide* (77%) as white crystals; NMR δ (60 MHz; $CDCl_3$) 3.35 (3H, s, OCH_3), 4.85 (2H, d, J 6 Hz, NCH_2O), 7.40 (2H, d, J 8 Hz) and 7.85 (2H, d, J 8 Hz) (ArH) and 8.0 (1H, br, NH). Treatment of a solution of *N*-(acetoxymethyl)-4-chlorobenzamide in dichloromethane with excess ethanethiol and *N,N*-diisopropylethylamine, followed by washing with water, drying (Na_2SO_4) and evaporation of the solvent under reduced pressure afforded *4-chloro-N-(ethylthiomethyl)benzamide* (85%) as a white powder; NMR δ (60 MHz; $CDCl_3$) 1.30 (3H, t, J 7 Hz, CH_3), 2.70 (2H, q, J 7 Hz, CH_2CH_3), 4.50 (2H, d, J 6 Hz, NCH_2O), 7.45 (2H, d, J 8 Hz) and 7.85 (2H, d, J 8 Hz) (ArH) and 8.65 (1H, t, J 6 Hz, NH). Similarly, *N*-(acetoxymethyl)-4-chlorobenzamide was allowed to react at ambient temperature with excess diethylamine in dichloromethane to give *4-chloro-N-(diethylaminomethyl)benzamide* (86%) as an off-white solid; NMR δ (60 MHz; $CDCl_3$) 1.10 (6H, t, J 7 Hz, CH_3), 2.58 (4H, q, J 7 Hz, CH_2CH_3), 4.35 (2H, d, J 6 Hz, NCH_2N), 7.2 (1H, br, NH), 7.30 (2H, d, J 8 Hz) and 7.75 (2H, d, J 8 Hz) (ArH).

N-(Acetoxymethyl)-4-chlorobenzamide (0.5 mM) was incubated in Earl's buffer, pH 7.4, (2.5 ml) with $K^{14}CN$ (1 mM, specific activity 3.2 mCi/mmol) at 37° for 1 hr under shaking. Incubations of *N*-(acetoxymethyl)-4-chlorobenzamide (1 mM) with glutathione (10 mM) were performed in buffer containing 3-(*N*-morpholino)propanesulphonic acid (MOPS, 0.4 M, pH 7.4).

In vitro *metabolism of 4-chloro-(N-hydroxymethyl)benzamide.* Livers were excised and a homogenate (25%) was prepared in sucrose solution (2.5 M, pH 7.4). Microsomes were obtained in the standard way involving centrifugation of the homogenate at 9000 *g* for 20 min and centrifugation of the 9000 *g* supernatant at 100,000 *g* for 1 hr. 4-Chloro-*N*-(hydroxymethyl)benzamide (1 mM) was incubated either with microsomes or microsomal supernatant equivalent to 0.3 g liver. Microsomal incubations were carried out for 1 hr in Earl's buffer (2.5 ml) containing $MgCl_2$ (5 mM) and a NADPH generating system (NADP, 1 mM, glucose-6-phosphate 4.5 mM, glucose-6-phosphate dehydrogenase 1.5 units). Incubations with microsomal supernatant were performed in MOPS buffer (0.4 M). These incubates also contained either $K^{14}CN$ (1 mM, specific activity 3.2 mCi/mmol) or glutathione (10 mM). In some experiments, a system which generated 3'-phosphoadenosine-5'-phosphosulphate (PAPS; K_2SO_4 2.4 mM, ATP, 8 mM, mercaptoethanol, 2.4 mM) or acetyl-CoA (2.5 mM) with ATP (5 mM) was added to the incubation. Incubations were terminated either by placing incubates on ice, or by precipitation of proteins via addition of 20% aqueous zinc sulphate (0.6 ml) followed by saturated aqueous barium hydroxide (0.6 ml). For the analysis of 4-chloro-*N*-(cyanomethyl)benzamide the incubation mixtures were passed through C_{18} Sep-Pak cartridges (Waters Associates, Northwich, U.K.), which were washed with water (5 ml) and eluted with methanol (5 ml). The methanol extract was evaporated to dryness under partial vacuum in a Savants Speed-vac concentrator. For the analysis of *S*-(4-chlorobenzamidomethyl)glutathione, the incubates were centrifuged after denaturation of proteins and aliquots of the supernatant were used immediately for analysis.

HPLC analysis. Separation of metabolites was performed using an Altex 100A pump (Anachem Ltd., Luton, U.K.) linked to a Pye LC-UU detector (λ = 254 nm) (Pye Unicam, Cambridge, U.K.) or a Waters trimodular system both fitted with Waters RCM-100 radial compression units and C_{18} 5-m reverse phase columns. For the analysis of the cyanomethylamide, the mobile phase was 60% methanol–40% 0.05 M sodium acetate in water (adjusted to pH 5); for the analysis of *S*-(4-chlorobenzamidomethyl)glutathione: 50% methanol–50% 0.05 M aqueous sodium acetate or 60% methanol–40% (1% trifluoroacetic acid in water). The flow rate was 1 ml/min.

Bacterial mutagenicity tests. The benzamide derivatives were evaluated using the plate incorporation assay as described by Maron and Ames [12] with *Salmonella typhimurium* strains TA 98 and TA 100. Some tests of 4-chloro-*N*-(hydroxymethyl)-benzamide involved preincubation of the compound with a liver 9000 *g* supernatant (10%) obtained from rats treated with Aroclor with or without K_2SO_4 (2.4 mM) and ATP (8 mM) or acetyl-CoA (2.5 mM) and ATP (5 mM) preceding the addition of the bacteria. The inclusion of acetyl-CoA was prompted by the observation that the mutagenicity of metabolites of the herbicide Monuron, a *N*-methyl compound related to the *N*-methylbenzamides, was increased in the presence of this cofactor [6].

RESULTS

Under physiological conditions, 4-chloro-*N*-(hydroxymethyl)benzamide did not react either with cyanide to afford the cyanomethylamide (Fig. 1) or with glutathione to yield the *S*-amidomethyl glutathione derivative (Fig. 2). In contrast, the acetate ester reacted with both cyanide (Fig. 1) and glutathione (Fig. 2). The product of the reaction between *N*-(acetoxymethyl)-4-chlorobenzamide and glutathione was isolated by HPLC. High-field proton NMR showed this material to be *S*-(4-chlorobenzamidomethyl)glutathione consistent with the spectrum of a sample of the compound prepared by the general method previously reported [11]. Under the incubation conditions used, 3% of the *N*-acetoxymethyl compound reacted with cyanide to give the cyanomethylamide, whereas the reaction with glutathione was almost quantitative.

In order to investigate the mechanisms whereby *N*-(acetoxymethyl)-4-chlorobenzamide reacts with nucleophiles, several experiments were conducted. Firstly, the pH dependence of the rate of hydrolysis of this compound to 4-chloro-*N*-(hydroxymethyl)benzamide in 0.02 M phosphate buffer was determined at 37° by HPLC analysis. The $t_{1/2}$ of hydrolysis was 13.2 min at pH 3, 16.9 min at pH 7 and less than 1 min at pH 9, which shows that the reaction was base-catalysed. Secondly, the substrate, in methanol, was treated with triethylamine. No reaction was observed by TLC during 10 min in the absence of base. Addition of successive aliquots of triethylamine caused very rapid methanolysis involving stoichiometric consumption of base. The isolated product was 4-chloro-*N*-(methoxymethyl)benzamide (see Fig. 4) in virtually quantitative yield, hence the electrophilic reactivity of the starting ester is at the methylene carbon exclusively rather than at the ester carbonyl. The reaction is stoichiometric in base, rather than catalytic. A similar reaction of *N*-(acetoxymethyl)-4-chlorobenzamide was observed with diethylamine and with ethanethiol in the presence of the non-nucleophilic base *N*,*N*-di*iso*propylethylamine, giving the diethylaminomethyl- and ethylthiomethylbenzamides, respectively. Under these experimental conditions, 4-chloro-*N*-(hydroxymethyl)benzamide did not react with methanol, ethanethiol or diethylamine.

When 4-chloro-*N*-(hydroxymethyl)benzamide was incubated with either mouse hepatic microsomes or microsomal supernatant with or without addition of acetyl CoA or a PAPS generating system, metabolism to precursors of methylene iminium ions or imines did not occur as indicated by the lack of evidence for the formation of a cyanomethylamide in the presence of cyanide or a glutathionyl derivative in the presence of glutathione (results not shown).

Whether *N*-(acetoxymethyl)-4-chlorobenzamide reacts with glutathione *in vivo* is unclear. The urine of mice which had received *N*-(acetoxymethyl)-4-

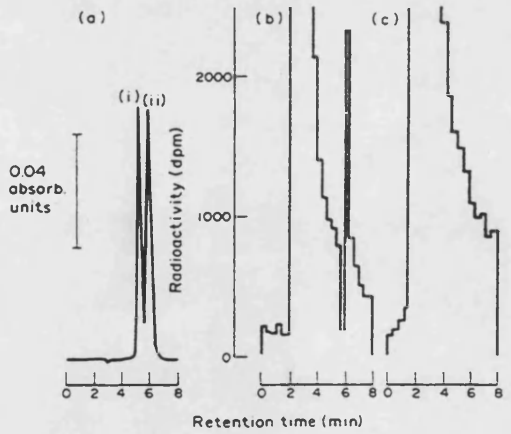

Fig. 1. High pressure liquid chromatogram of (a) reference compounds 4-chloro-*N*-(hydroxymethyl)benzamide (i) and 4-chloro-*N*-(cyanomethyl)benzamide (ii); (b) a sample of the incubate of *N*-(acetoxymethyl)-4-chlorobenzamide with $K^{14}CN$; and (c) a sample of the incubate of 4-chloro-*N*-(hydroxymethyl)benzamide with $K^{14}CN$. Detection by u.v. spectrophotometry (a) or radioactivity counting (b and c). Traces are representative of three experiments.

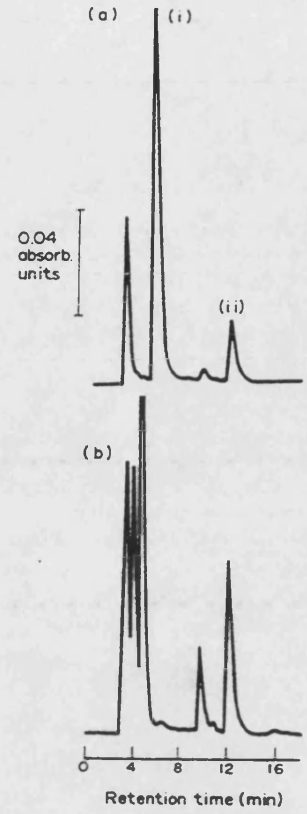

Fig. 2. High pressure liquid chromatogram of (a) a sample of the incubate of *N*-(acetoxymethyl)-4-chlorobenzamide with glutathione; (b) a sample of the incubate of 4-chloro-*N*-(hydroxymethyl)benzamide with a 9000 *g* supernatant and glutathione. Peak (i) co-elutes with *S*-(4-chlorobenzamidomethyl)glutathione and peak (ii) with 4-chloro-*N*-(hydroxymethyl)benzamide. A chromatographic trace very similar to the one shown in (b) was observed when the 9000 *g* supernatant was fortified with a PAPS generating system.

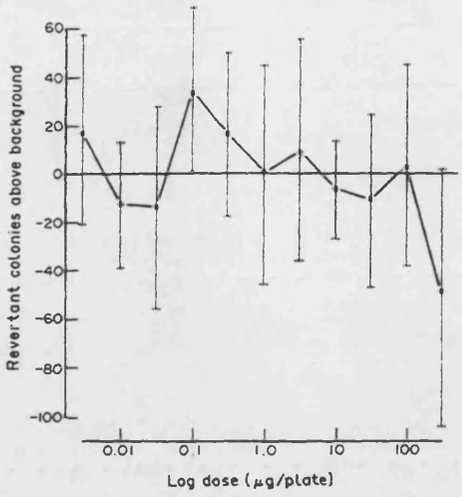

Fig. 3. Lack of mutagenicity of *N*-(acetoxymethyl)-4-chlorobenzamide to *Salmonella typhimurium* TA 100. Results are the mean ± SD of three plates.

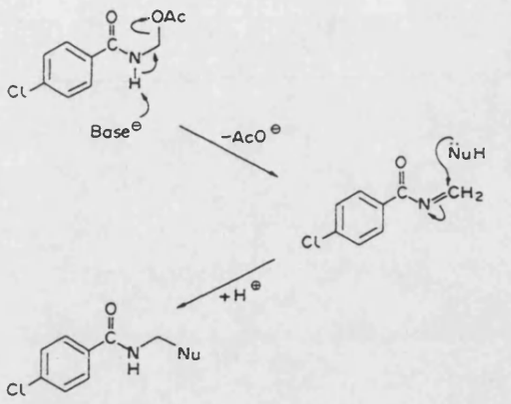

Fig. 4. Mechanism of the base-catalysed reaction between *N*-(acetoxymethyl)-4-chlorobenzamide and nucleophiles (Nu = CN^-, glutathione, MeO, EtS, Et_2N).

chlorobenzamide or 4-chloro-*N*-(hydroxymethyl)-benzamide contained 4-chlorohippuric acid and the HPLC chromatograms showed a peak which was possibly due to *S*-(4-chlorobenzamidomethyl)-*N*-acetylcysteine (results not shown). The small amount of this material formed and its chromatographic properties rendered its identification impossible.

Neither 4-chloro-*N*-(hydroxymethyl)benzamide nor its acetyl ester were genotoxic in the *Salmonella* assay (TA 100 strain) in the presence or absence of a 9000 *g* supernatant fraction obtained from rat (Table 1, Fig. 3). *N*-(Acetoxymethyl)-4-chlorobenzamide was, however, much more toxic towards the bacteria than was 4-chloro-*N*-(hydroxymethyl)-benzamide. At a concentration of 0.316 mg per plate of the acetate ester, 50% of the bacteria were killed whereas a concentration of 5 mg per plate 4-chloro-*N*-(hydroxymethyl)benzamide did not cause toxicity to the bacteria. Results obtained with the TA 98 strain were very similar to those observed with TA 100, with the rate of spontaneous reversion similar to that reported previously [12].

DISCUSSION

The results of this study demonstrate unequivocally that 4-chloro-*N*-(hydroxymethyl)benzamide does not form a methyleneiminium or methyleneimine under physiological conditions. This is not unexpected since the hydroxide group is a poor leaving group. However, the situation is very different in a molecule in which the *N*-hydroxymethyl group is esterified. In the model compound, *N*-(acetoxymethyl)-4-chlorobenzamide, the acetoxy group is a good leaving group. This renders the molecule capable of forming an imine under physiological conditions. Such species can react with cyanide to yield the cyanomethylamide or with glutathione to afford *S*-(4-chlorobenzamidomethyl)glutathione. In the presence of cyanide, the elimination–addition reaction with the electrophilic methylene was not the only reaction occurring, in that cyanide appeared

Table 1. Lack of mutagenicity of 4-chloro-*N*-(hydroxymethyl)benzamide to *Salmonella typhimurium* strain TA 100

Dose (μg/plate)	Number of revertant colonies above background, in the presence of 9000 *g* fraction	9000 *g* fraction and PAPS generating system	9000 *g* fraction and acetyl-CoA and ATP
50	−9*	3	16
100	−9 ± 22	−29 ± 25	−11 ± 21
500	−18 ± 16	−30	−21 ± 8
1000	20 ± 6	−29 ± 18	−42
5000	−12 ± 33	−21	−5
2-Acetylaminofluorene 50 μg/plate	2073 ± 219		

* Values are the mean of two or the mean ± SD of 3–5 tests. Background levels were: 123 ± 3 (9000 *g* supernatant), 98 ± 18 (9000 *g* fraction and PAPS generating system), 81 ± 18 (9000 *g* fraction and acetyl-CoA).

also to catalyse hydrolysis by acting as a base as well as a nucleophile. Therefore, the amount of the cyanomethylamide formed in the presence of cyanide was much less than the amount of the glutathionyl derivative generated during the reaction of the ester with glutathione. The pH dependence and the stoichiometry of base consumption of these substitution reactions suggests a mechanism implicating the methyleneimine rather than the methyleneiminium, as outlined in Fig. 4.

The results indicate that the metabolic conjugation of 4-chloro-*N*-(hydroxymethyl)benzamide with acetyl-CoA, activated sulphate or UDP-glucuronic acid is not a biochemical reaction *in vitro*. Reaction products of the *N*-hydroxymethyl compound with cyanide or glutathione could not be detected in incubations of liver fractions supplemented with either acetyl-CoA or a PAPS generating system. Furthermore, the presence of small amounts of a mercapturate derived from 4-chloro-*N*-(hydroxymethyl)benzamide was suspected in the urine of mice which had received this carbinolamide, but unequivocal identification was not possible. These observations are in accordance with our failure to detect either *S*-(*N*-(2,4,6-bis(dimethylamino)-1,3,5-triazin-5-yl)-*N*-methylaminomethyl)glutathione as a metabolite of *N*-(hydroxymethyl)pentamethylmelamine *in vitro* in liver preparations or *N*-acetyl-*S*-(*N*-(2,4,6-bis-(dimethylamino)-1,3,5-triazin-5-yl)-*N*-methylaminomethyl)cysteine as an *in vivo* metabolite of this compound in the urine of mice.*

Two recent reports describe the identification of NCH_2S conjugates of *N*-methyl compounds *in vivo*. A major biliary metabolite of the hepatocarcinogen 4-dimethylaminoazobenzene in the rat was shown to be *S*-((4-phenylazo)phenylaminomethyl)glutathione [7] and a major urinary metabolite of 4-cyano-*N*,*N*-dimethylaniline has been identified as *N*-acetyl-*S*-((4-cyanophenyl)aminomethyl)cysteine in rodents [8]. In both cases, the thioester metabolites were considered to be the products of the reaction between glutathione and either methyleneiminium ions or imines derived from the *N*-hydroxymethyl intermediates. As the hydroxymethyl moiety in the aniline derivatives is located in an environment of greater electron density than in the *N*-(hydroxymethyl)benzamide molecule, it is reasonable to assume that they undergo facile elimination of water, as do the aliphatic *N*-(hydroxymethyl)amines [13]. Alternatively, one could suggest that, in analogy to the results reported in this paper, metabolic esterification increases the leaving group ability of the hydroxy group in these *N*-(hydroxymethyl)aniline derivatives.

4-Chloro-*N*-(hydroxymethyl)benzamide was not a mutagen in the *Salmonella* mutation assay. This is not surprising in the light of its inability to form electrophilic species. It is, however, puzzling that *N*-(acetoxymethyl)-4-chlorobenzamide, which we have now shown unequivocally to be a precursor of electrophilic species, is not mutagenic. Two explanations can be tendered: either the methyleneimine formed by elimination of acetate is intrinsically unreactive with DNA to cause deleterious lesions or, more likely, the *Salmonella* test is incapable of detecting the potential mutagenicity of this kind of soft electrophile. It has been shown recently that *N*-(hydroxymethyl)chloroacetamide, an amide related to 4-chloro-*N*-(hydroxymethyl)benzamide, was without genotoxic properties in *Salmonella* [14]. However, it was shown to be clastogenic in a human lymphocyte cytogenic assay, both in the presence and absence of rodent liver 9000 *g* homogenate. It remains to be investigated whether or not *N*-(acetoxymethyl)-4-chlorobenzamide or, indeed, 4-chloro-*N*-(hydroxymethyl)benzamide are clastogenic in this genotoxicity test.

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* D. Ross, A. Gescher and M. D. Threadgill, unpublished results.

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The Role of Oxidative Processes in the Cytotoxicity of Substituted 1,4-Naphthoquinones in Isolated Hepatocytes

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In order to clarify the role of oxidative processes in cytotoxicity we have studied the metabolism and toxicity of 2-methyl-1,4-naphthoquinone (menadione) and its 2,3 dimethyl (DMNQ) and 2,3 diethyl (DENQ) analogs in isolated rat hepatocytes. The two analogs, unlike menadione, cannot alkylate nucleophiles directly and were considerably less toxic than menadione. This decreased toxicity was consistent with the inability of DMNQ and DENQ to alkylate but we also found them to undergo lower rates of redox cycling in hepatocytes and a higher ratio of two electron as opposed to one electron reduction relative to menadione. Thus, facile analysis of the respective roles of alkylation and oxidation in cytotoxicity was not possible using these compounds. In hepatocytes pretreated with bischloroethyl-nitrosourea (BCNU) to inhibit glutathione reductase, all three naphthoquinones caused a potentiation of reduced glutathione (GSH) removal/oxidized glutathione (GSSG) generation and cytotoxicity relative to that observed in control cells. These data show that inhibition of hepatocyte glutathione reductase by BCNU results in enhanced naphthoquinone-induced oxidative challenge and subsequent cellular toxicity. That DMNQ and DENQ are cytotoxic, albeit at high concentrations, and that this cytotoxicity is potentiated by BCNU pretreatment suggest that oxidative processes alone can be a determinant of cytotoxicity.

The metabolism and toxicity of menadione,[2] 2-methyl-1,4-naphthoquinone, have been extensively studied in isolated hepatocytes (1-5) which constitutes a useful model system for investigations of mechanisms of hepatotoxicity *in vitro*. Menadione-induced toxicity in hepatocytes is thought to involve depletion of cellular thiols such as glutathione (GSH), and thiol groups contained in proteins (4, 5). Recent evidence has implicated removal of cellular thiol groups to be the mechanism whereby menadione-induced perturbations in intracellular calcium ion homeostasis may occur (5, 6). These changes may lead to increases in cytosolic free calcium concentration, cytoskeletal alterations, and subsequent cell death (1, 2).

The removal of cellular thiols in cells by menadione is a consequence of two major

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[2] Abbreviations used: Menadione, 2-methyl-1,4-naphthoquinone; DMNQ, 2,3-dimethyl-1,4-naphthoquinone; DENQ, 2,3-diethyl-1,4-naphthoquinone; DT-diaphorase, NAD(P)H: (quinone acceptor) oxidoreductase; SOD, superoxide dismutase; BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMSO, dimethylsulfoxide.

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processes, alkylation and oxidation. Menadione is an electrophile and as such can react with nucleophiles directly, generating a menadione–GSH conjugate and alkylated protein in the case of GSH and protein SH groups, respectively (4, 7). Menadione is also a substrate for various one-electron reductases in both the mitochondrial and endoplasmic reticular compartments of the cell (1). These enzymes catalyze the production of semiquinone radicals derived from menadione whose subsequent reoxidation by molecular oxygen generates the parent quinone and superoxide anion radical ($\dot{O}_2^-$) (1). Dismutation of $\dot{O}_2^-$ produces hydrogen peroxide (H_2O_2), and further reactions of these species can lead to the production of hydroxyl radical (OH) and singlet oxygen ($^1O_2$) (7, 8). Thus, an assortment of oxidizing species can be produced in the cell as a consequence of one-electron reduction of menadione, and such species will readily oxidize reduced thiols.

In an attempt to clarify the role of the generation of oxidizing species in quinone-induced cytotoxicity, we have studied the metabolism of menadione and two of its analogs which cannot alkylate nucleophilic sites directly. In this study we have compared the metabolism and toxicity of menadione, 2,3-dimethyl-1,4-naphthoquinone (DMNQ) and 2,3-diethyl-1,4-naphthoquinone (DENQ), in isolated rat hepatocytes. Furthermore, we have also investigated the role of oxidative processes in the cytotoxicity of these three naphthoquinones by using bis-chloroethylnitrosourea (BCNU) to inhibit cellular glutathione reductase (9). This enzyme, which utilizes NADPH as a cofactor, reduces GSSG to GSH, thus ensuring a high intracellular ratio of reduced to oxidized thiol. Inhibition of glutathione reductase severely limits the efficient functioning of glutathione peroxidase, an enzyme which removes peroxides such as H_2O_2 at the expense of the conversion of GSH to GSSG. Thus H_2O_2 produced, for example, from the dismutation of $\dot{O}_2^-$, can only be removed by this enzyme until the initial supply of GSH is exhausted, as the catalytic cycle regenerating GSH via glutathione reductase is blocked. We have compared the toxicity of menadione, DMNQ, and DENQ to both control and BCNU pretreated hepatocytes and measured the effects of the quinones on cellular thiol homeostasis.

MATERIALS AND METHODS

1,4-Naphthoquinone was purchased from Aldrich. GSH, GSSG, dicoumarol, and menadione were obtained from Sigma, while collagenase (grade II), NADPH, and superoxide dismutase (SOD) were purchased from Boehringer-Mannheim. All other chemicals were of at least reagent grade and were purchased locally.

2,3-Dimethyl-1,4-naphthoquinone (DMNQ) and 2,3-diethyl-1,4-naphthoquinone (DENQ) were prepared as follows: a mixture of 1,4-naphthoquinone (1.58 g), propanoic acid (6.0 g), sulpholane (20 ml), water (25 ml), and silver nitrate (0.5 g) was heated to 80°C. Potassium peroxydisulfate (5.5 g) in water (25 ml) was added during 20 min at 80°C. After a further 15 min at this temperature, the mixture was poured onto ice (100 g). The water was decanted off and the residue dissolved in acetonitrile (50 ml), filtered, and evaporated to dryness. The residue was chromatographed on Silica gel, with petroleum ether (BPt 60–80°C)/ethyl acetate (9/1) giving 2,3-diethyl-1,4-naphthoquinone (1.61 g; 75%; MPt 67–68°C, lit. MPt 68.5–69.5°C (10)). An analogous preparation was used for 2,3-dimethyl-1,4-naphthoquinone, using 1,4-naphthoquinone and acetic acid, in 79% yield (MPt 124–125°C; lit. MPt 125–126°C (11)). TLC was used to check the purity of these compounds (Silica plates; petroleum ether/ethyl acetate 3/1) DMNQ-one spot R_f = 0.85; DENQ-one spot R_f = 0.90. NMR analysis was totally consistent with the proposed structures.

Male Sprague–Dawley rats (200–250 g) were used in all experiments. Hepatocytes were isolated as in (12) and cell incubations were performed in rotating round-bottomed flasks (10^6 cells/ml) under an atmosphere of 95% O_2 5% CO_2. Glutathione reductase was inhibited by treatment of the cells with 1,3-bis-(2-chloroethyl)-1-nitrosourea essentially as previously described (13), except that Eagles minimal essential medium supplemented with 1 mM methionine was used for cell treatment and cell recovery. This treatment produces at least 90% inhibition of GSSG reductase without affecting cellular GSH content (13) and this was verified in these studies. Incubations were performed in Krebs–Henseleit buffer, pH 7.4, containing 12.5 mM Hepes at 37°C, and substrates were added in dimethylsulfoxide (0.4% of final volume except for toxicity curves of DMNQ and DENQ 0.6 mM (0.8%) and 1 mM (2%)). Control experiments showed that concentrations of up to 2% DMSO were not cytotoxic and had no effect on menadione-induced toxicity. Cell viability was determined by trypan blue exclusion. GSH and GSSG were determined by HPLC after de-

rivatization as described by Reed *et al.* (14). $\bar{O}_2^{\cdot}$ was detected by SOD-sensitive reduction of acetylated cytochrome *c*, prepared as in (15) using the wavelength pair 550–540 nm (16). Dialyzed whole liver homogenate was prepared by homogenization of the liver in 0.25 M sucrose (20% w/v), centrifugation at 2000*g* for 10 min, followed by overnight dialysis of the supernatant against 0.15 M KCl, pH 7.4 (21). Aliquots of supernatant (0.05 ml) were mixed with 0.15 M Tris-HCl buffer, pH 7.4 (1 ml), containing acetylated cytochrome *c* (1 mg/ml) and NADPH, 1 mM. In all experiments dicoumarol was used at a concentration of 30 μM and SOD at 0.1 mg/ml. SOD-sensitive reduction of ferricytochrome derivatives cannot be used as an absolute measure of $\bar{O}_2^{\cdot}$ production due to other competing reactions of $\bar{O}_2^{\cdot}$, such as enzymatic and nonenzymatic dismutation. Protein was determined according to the method of Lowry *et al.* (17).

RESULTS

Alkylation reactions of quinones generally proceed at the ortho position to the quinone moiety. Thus both the 2,3-dialkylated derivatives (DMNQ and DENQ) would not be expected to directly alkylate biological nucleophiles. This was verified experimentally using GSH as a model nucleophile. If conjugate formation between quinone and GSH did not occur then full recovery of thiol equivalents in terms of GSH remaining and GSSG formed should be possible. That this was the case during reaction of DMNQ and DENQ with GSH is shown in Table I.

The toxicity of menadione and its two alkylated analogs, DMNQ and DENQ, in isolated rat hepatocytes is shown in Fig. 1 (A–C). The results show that both of the substituted analogs were markedly less toxic than menadione. The lack of toxicity of DMNQ and DENQ could reflect their inability to alkylate cellular nucleophiles but may also be a consequence of diminished one-electron reduction by mitochondrial and microsomal reductases relative to menadione. This would in turn lead to a decreased generation of semiquinone radical, and active oxygen species such as $\bar{O}_2^{\cdot}$ and H_2O_2. Alternatively, the diminished toxicity of DMNQ and DENQ may be due to their higher affinity for the enzyme DT-diaphorase [NADPH (quinone acceptor) oxidoreductase], a two-electron reductase which catalyzes the reduction of quinones to hydroquinones without the generation of semiquinone radical intermediates (18). Thus, it has been proposed that this enzyme plays a protective role in quinone-induced toxicity (1, 19). A dialyzed whole liver homogenate, devoid of soluble cellular thiols (which may interact with quinones directly to generate $\bar{O}_2^{\cdot}$ (4, 20)), was used to investigate these possibilities. A decreased capacity of DMNQ and DENQ relative to menadione to generate species which caused acetylated cytochrome *c* reduction was evident using the dialyzed whole liver homogenate preparation (Fig. 2A). Reduction of the cytochrome can reflect generation of either hydroquinone[3] or $\bar{O}_2^{\cdot}$, as both

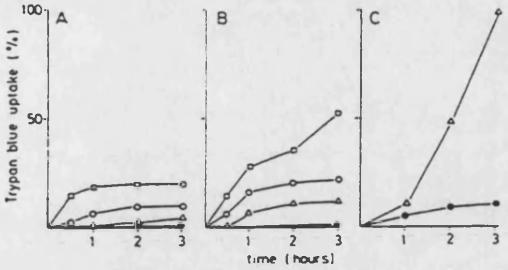

FIG. 1. Toxicity of 2,3-dimethyl-1,4-naphthoquinone (A), 2,3-diethyl-1,4-naphthoquinone (B), and menadione (C) in isolated hepatocytes. (Quinone concentrations were: ●, 0.1 mM; △, 0.2 mM; ○, 0.6 mM, □, 1 mM). One experiment typical of three.

TABLE I

RECOVERY OF THIOL EQUIVALENTS AFTER REACTION OF DMNQ OR DENQ (0.2 mM) WITH GSH (0.2 mM) FOR 30 min AT 37°C[a]

	GSH remaining (μM)	GSSG formed (μM GSH eq)	Total (GSH eq)
Without quinone	170	31	201
DMNQ	143	66	209
DENQ	138	66	204

[a] In Krebs-Henseleit buffer, pH = 7.4.

[3] We have found that the hydroquinone of menadione, synthesized according to L. Feiser [(1940) *J. Biol. Chem.* 133, 391–396], readily reduces acetylated cytochrome *c*.

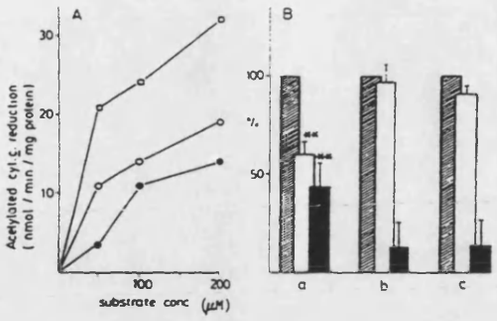

FIG. 2. NADPH-dependent reduction of acetylated cytochrome *c* induced by 1,4-naphthoquinones in dialyzed whole liver homogenate. (Menadione (□); 2,3-dimethyl-1,4-naphthoquinone (○); and 2,3-diethyl-1,4-naphthoquinone (●)). (A) Concentration dependence of NADPH-(1 mM) dependent acetylated cytochrome *c* reduction in dialyzed whole liver homogenate. (B) Effects of SOD (0.2 mg/ml, open bar), and dicoumarol (0.03 mM, solid bar) on NADPH-dependent reduction of acetylated cytochrome *c* in dialyzed whole liver homogenate. (a) Menadione, 0.2 mM; (b) 2,3-dimethyl-1,4-naphthoquinone, 0.2 mM; (c) 2,3-diethyl-1,4-naphthoquinone, 0.2 mM. Means ± standard deviation of three experiments.

are capable of transferring electrons to acetylated cytochrome *c*. (1). With menadione, however, 50% of the reduction was SOD sensitive, suggesting the involvement of $\bar{O}_2^{\cdot}$, whereas SOD had no significant effect on DMNQ- and DENQ-induced acetylated cytochrome *c* reduction (Fig. 2B). Conversely, dicoumarol, an inhibitor of DT-diaphorase (19), almost totally inhibited the NADPH-dependent reduction of the cytochrome by DMNQ and DENQ, whereas menadione-induced reduction was still 35% of the control value (Fig. 2B). These experiments demonstrate that the alkyl-substituted analogs of menadione have a higher ratio of two- as opposed to one-electron reduction relative to menadione itself. In agreement with previous studies (1, 21) and the protective role of DT-diaphorase, pretreatment of hepatocytes with dicoumarol (0.03 mM) markedly exacerbated the toxicity of all three quinones to hepatocytes (data not shown).

In an attempt to clarify the role of oxidative processes in naphthoquinone-induced toxicity we have used cells pretreated with BCNU, an inhibitor of glutathione reductase. This treatment exacerbates intracellular oxidative challenge, as any GSSG generated in the cell cannot be reduced back to GSH. Both DMNQ and DENQ caused some loss in hepatocyte GSH content (Figs. 3B–C) relative to controls (Fig. 3A). There was, however, a potentiation of GSH removal of GSSG generation in BCNU-pretreated cells relative to control cells in the case of all three naphthoquinones (Figs. 3A–D). Furthermore, the three naphthoquinones were significantly more toxic to BCNU-pretreated cells than to control cells (Figs. 4A–D), demonstrating that enhanced oxidative challenge can result in enhanced cellular toxicity.

DISCUSSION

In this study we have investigated the metabolism and toxicity of alkyl-substituted analogs of menadione which cannot alkylate cellular nucleophiles directly in order to determine the role of oxidative processes in cytotoxicity. The alkyl-substituted analogs of menadione, DMNQ and DENQ, were markedly less toxic to isolated hepatocytes than the parent compound. High concentrations of DMNQ and DENQ were required to induce significant cytotoxicity in hepatocytes. These data do not allow us to conclude, however, that oxidative processes are unimportant in menadione-induced cytotoxicity and that alkylation reactions represent the only determinants of the toxicity observed. An alternative explanation for the decreased toxicity of DMNQ and DENQ relative to menadione may be their low ability to generate oxidizing species within the cell—either because of low affinity for one-electron reductases or because of higher affinity for alternative pathways of metabolism such as DT-diaphorase-catalyzed two-electron reduction. Using dialyzed liver homogenate we found that SOD could substantially inhibit menadione-induced acetylated cytochrome *c* reduction, whereas it had minimal inhibitory effect on DMNQ- and DENQ-mediated reduction (Fig. 2). This suggests that $\bar{O}_2^{\cdot}$ generated by redox cycling reactions is responsible for a substantial proportion of the observed reduction of

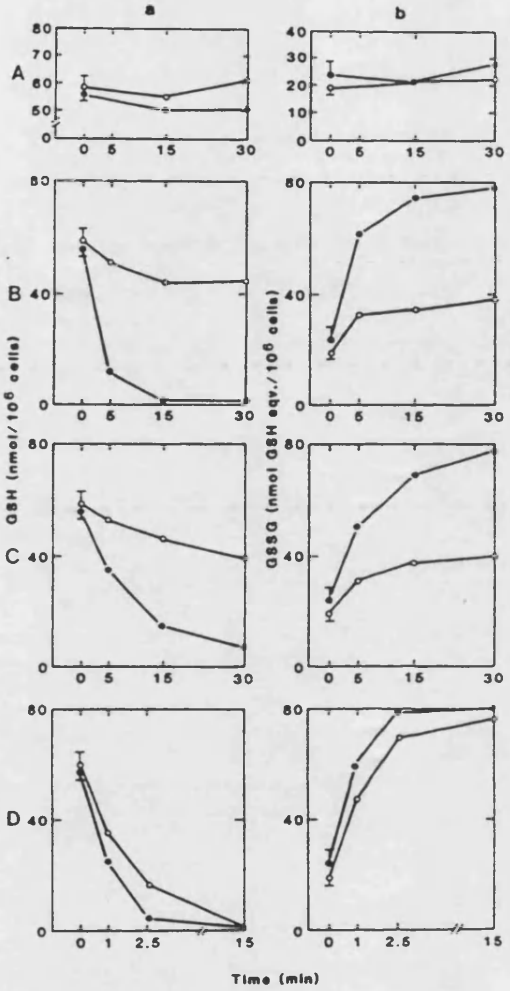

FIG. 3. GSH depletion (a) and GSSG generation (b) during metabolism of 2,3-dimethyl-1,4-naphthoquinone 0.2 mM (B), 2,3-diethyl-1,4-naphthoquinone 0.2 mM (C), and menadione 0.05 mM (D), in control (○) and BCNU-pretreated cells (●). The thiol status of control (○) and BCNU-pretreated (●) cells in the absence of quinone but in the presence of the solvent vehicle (DMSO) is shown in (A). One experiment typical of three.

acetylated cytochrome *c* only with menadione. Furthermore, data obtained using dicoumarol, an inhibitor of DT-diaphorase, showed that two-electron reduction was involved in the metabolism of all three naphthoquinones, but that this metabolic pathway was more active with DMNQ and DENQ. The alkylated analogs, therefore, undergo a higher ratio of two- as opposed to one-electron reduction relative to menadione and thus this represents a possible explanation of the decreased toxicity of DMNQ and DENQ relative to menadione.

Whether a particular quinone is metabolized by one- or two-electron reductases depends on a number of factors such as steric considerations, lipophilicity, and one-electron reduction potential. The dependence of the rate of $\bar{O}_2^{\cdot}$ production by isolated rat hepatocytes on the one-electron reduction potential of quinones has been studied previously by Powis *et al.* (22). These workers showed that in isolated hepatocytes maximal quinone-induced $\bar{O}_2^{\cdot}$ formation occurs at a quinone one-electron reduction potential of −70 mV (22). From their data one can also predict that a more negative one-electron reduction potential

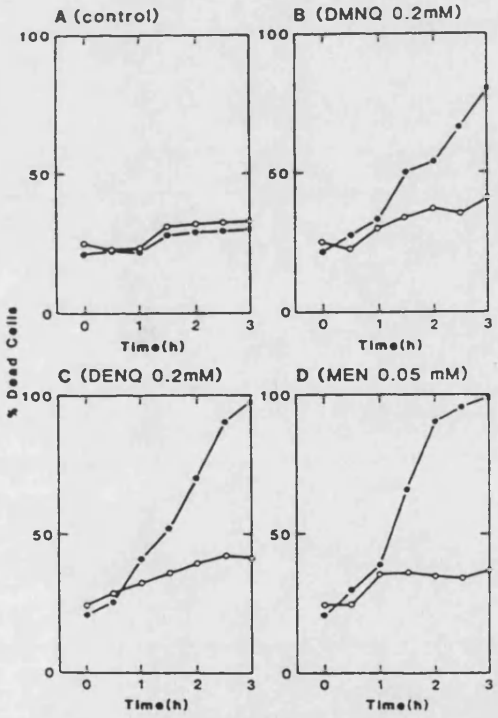

FIG. 4. Toxicity of 2,3-dimethyl-1,4-naphthoquinone 0.2 mM (B), 2,3-diethyl-1,4-naphthoquinone 0.2 mM (C), and menadione 0.05 mM (D), in control (○) and BCNU-pretreated cells (●). The toxicity of the solvent vehicle to control (○) and BCNU-pretreated (●) cells is shown in (A). One experiment typical of five.

than that of menadione (−200 mV) would lead to a marked decrease in the rate of superoxide formation. As alkyl substitution of the naphthoquinone nucleus would be expected to cause donation of electrons into the ring system, the one-electron reduction potentials of DMNQ and DENQ would be predicted to be more negative than menadione itself. Thus, on the basis of one-electron reduction potential alone, the alkyl-substituted derivatives of menadione would be expected to induce the generation of fewer oxygen-derived radicals.

The observation that DMNQ and DENQ were cytotoxic, albeit at high concentrations, suggests that oxidative processes alone can cause cytotoxicity. To investigate this we used cells preincubated with BCNU, which inhibits cellular glutathione reductase. In control cells, all three naphthoquinones induced some GSH loss and GSSG formation, but these changes were markedly exacerbated by BCNU pretreatment (Fig. 3), showing that the alterations in GSH homeostasis observed occur, at least in part, by means of oxidative mechanisms. Although DMNQ and DENQ did not induce significant formation of $\dot{O}_2^-$ in dialyzed liver homogenate (Fig. 2), naphthoquinones can still induce an oxidative challenge including $\dot{O}_2^-$ and H_2O_2 generation, via chemical reactions (23), and this may represent the route whereby DMNQ and DENQ induce oxidative stress.

As well as a potentiation of naphthoquinone-induced GSH loss and GSSG formation in BCNU-pretreated cells relative to control cells, marked potentiation of toxicity in the nitrosourea-treated cells was also observed. It seems probable that the potentiation of oxidative challenge observed in BCNU-pretreated cells contributes to the increased toxicity of the naphthoquinones, although our data is not conclusive proof of this. It is clear from the data shown in Figs. 3D and 4D that GSH loss alone does not necessarily result in cytotoxicity, thus confirming previous work (5).

When both alkylation and oxidation can play a role in quinone-induced cytotoxicity, for example in the case of 1,4-naphthoquinone (21) and menadione (5), the dissection of the relative importance of each process for the eventual cytotoxic effect observed is not a simple exercise. Indeed, as the alkylation of thiols by menadione (4, 20) and 1,4-naphthoquinone (G. Cohen, personal communication) produces active oxygen species, then the two processes are not mutually exclusive. In addition naphthoquinones may induce cytotoxicity by other undefined processes. Thus, it seems obvious that in the case of menadione and 1,4-naphthoquinone more than one mechanism contributes to cytotoxicity.

In this study we have shown that derivatives of menadione which cannot directly alkylate nucleophiles are still cytotoxic, albeit at high concentrations, in isolated hepatocytes. We have also investigated the toxicity of naphthoquinones in BCNU-pretreated cells. Increased quinone-induced changes in soluble thiol oxidation were observed in BCNU-pretreated cells and these changes were associated with exacerbated cytotoxicity. These data suggest that oxidative processes alone can function as a determinant of the cytotoxicity of naphthoquinones.

ACKNOWLEDGMENTS

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PUBLICATION 20

The Role of Oxidative Processes in the Cytotoxicity of 1,4-Naphthoquinones in Isolated Hepatocytes

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The Role of Oxidative Processes in the Cytotoxicity of Substituted 1,4-Naphthoquinones in Isolated Hepatocytes

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In order to clarify the role of oxidative processes in cytotoxicity we have studied the metabolism and toxicity of 2-methyl-1,4-naphthoquinone (menadione) and its 2,3 dimethyl (DMNQ) and 2,3 diethyl (DENQ) analogs in isolated rat hepatocytes. The two analogs, unlike menadione, cannot alkylate nucleophiles directly and were considerably less toxic than menadione. This decreased toxicity was consistent with the inability of DMNQ and DENQ to alkylate but we also found them to undergo lower rates of redox cycling in hepatocytes and a higher ratio of two electron as opposed to one electron reduction relative to menadione. Thus, facile analysis of the respective roles of alkylation and oxidation in cytotoxicity was not possible using these compounds. In hepatocytes pretreated with bischloroethyl-nitrosourea (BCNU) to inhibit glutathione reductase, all three naphthoquinones caused a potentiation of reduced glutathione (GSH) removal/oxidized glutathione (GSSG) generation and cytotoxicity relative to that observed in control cells. These data show that inhibition of hepatocyte glutathione reductase by BCNU results in enhanced naphthoquinone-induced oxidative challenge and subsequent cellular toxicity. That DMNQ and DENQ are cytotoxic, albeit at high concentrations, and that this cytotoxicity is potentiated by BCNU pretreatment suggest that oxidative processes alone can be a determinant of cytotoxicity.

The metabolism and toxicity of menadione,[2] 2-methyl-1,4-naphthoquinone, have been extensively studied in isolated hepatocytes (1–5) which constitutes a useful model system for investigations of mechanisms of hepatotoxicity *in vitro*. Menadione-induced toxicity in hepatocytes is thought to involve depletion of cellular thiols such as glutathione (GSH), and thiol groups contained in proteins (4, 5). Recent evidence has implicated removal of cellular thiol groups to be the mechanism whereby menadione-induced perturbations in intracellular calcium ion homeostasis may occur (5, 6). These changes may lead to increases in cytosolic free calcium concentration, cytoskeletal alterations, and subsequent cell death (1, 2).

The removal of cellular thiols in cells by menadione is a consequence of two major

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[2] Abbreviations used: Menadione, 2-methyl-1,4-naphthoquinone; DMNQ, 2,3-dimethyl-1,4-naphthoquinone; DENQ, 2,3-diethyl-1,4-naphthoquinone; DT-diaphorase, NAD(P)H: (quinone acceptor) oxidoreductase; SOD, superoxide dismutase; BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMSO, dimethylsulfoxide.

processes, alkylation and oxidation. Menadione is an electrophile and as such can react with nucleophiles directly, generating a menadione-GSH conjugate and alkylated protein in the case of GSH and protein SH groups, respectively (4, 7). Menadione is also a substrate for various one-electron reductases in both the mitochondrial and endoplasmic reticular compartments of the cell (1). These enzymes catalyze the production of semiquinone radicals derived from menadione whose subsequent reoxidation by molecular oxygen generates the parent quinone and superoxide anion radical ($\bar{O}_2^{\cdot}$) (1). Dismutation of $\bar{O}_2^{\cdot}$ produces hydrogen peroxide (H_2O_2), and further reactions of these species can lead to the production of hydroxyl radical ($\dot{O}H$) and singlet oxygen ($^1O_2$) (7, 8). Thus, an assortment of oxidizing species can be produced in the cell as a consequence of one-electron reduction of menadione, and such species will readily oxidize reduced thiols.

In an attempt to clarify the role of the generation of oxidizing species in quinone-induced cytotoxicity, we have studied the metabolism of menadione and two of its analogs which cannot alkylate nucleophilic sites directly. In this study we have compared the metabolism and toxicity of menadione, 2,3-dimethyl-1,4-naphthoquinone (DMNQ) and 2,3-diethyl-1,4-naphthoquinone (DENQ), in isolated rat hepatocytes. Furthermore, we have also investigated the role of oxidative processes in the cytotoxicity of these three naphthoquinones by using bis-chloroethylnitrosourea (BCNU) to inhibit cellular glutathione reductase (9). This enzyme, which utilizes NADPH as a cofactor, reduces GSSG to GSH, thus ensuring a high intracellular ratio of reduced to oxidized thiol. Inhibition of glutathione reductase severely limits the efficient functioning of glutathione peroxidase, an enzyme which removes peroxides such as H_2O_2 at the expense of the conversion of GSH to GSSG. Thus H_2O_2 produced, for example, from the dismutation of $\bar{O}_2^{\cdot}$, can only be removed by this enzyme until the initial supply of GSH is exhausted, as the catalytic cycle regenerating GSH via glutathione reductase is blocked. We have compared the toxicity of menadione, DMNQ, and DENQ to both control and BCNU pretreated hepatocytes and measured the effects of the quinones on cellular thiol homeostasis.

MATERIALS AND METHODS

1,4-Naphthoquinone was purchased from Aldrich. GSH, GSSG, dicoumarol, and menadione were obtained from Sigma, while collagenase (grade II), NADPH, and superoxide dismutase (SOD) were purchased from Boehringer-Mannheim. All other chemicals were of at least reagent grade and were purchased locally.

2,3-Dimethyl-1,4-naphthoquinone (DMNQ) and 2,3-diethyl-1,4-naphthoquinone (DENQ) were prepared as follows: a mixture of 1,4-naphthoquinone (1.58 g), propanoic acid (6.0 g), sulpholane (20 ml), water (25 ml), and silver nitrate (0.5 g) was heated to 80°C. Potassium peroxydisulfate (5.5 g) in water (25 ml) was added during 20 min at 80°C. After a further 15 min at this temperature, the mixture was poured onto ice (100 g). The water was decanted off and the residue dissolved in acetonitrile (50 ml), filtered, and evaporated to dryness. The residue was chromatographed on Silica gel, with petroleum ether (BPt 60–80°C)/ethyl acetate (9/1) giving 2,3-diethyl-1,4-naphthoquinone (1.61 g; 75%; MPt 67–68°C, lit. MPt 68.5–69.5°C (10)). An analogous preparation was used for 2,3-dimethyl-1,4-naphthoquinone, using 1,4-naphthoquinone and acetic acid, in 79% yield (MPt 124–125°C; lit. MPt 125–126°C (11)). TLC was used to check the purity of these compounds (Silica plates; petroleum ether/ethyl acetate 3/1) DMNQ-one spot R_f = 0.85; DENQ-one spot R_f = 0.90. NMR analysis was totally consistent with the proposed structures.

Male Sprague-Dawley rats (200–250 g) were used in all experiments. Hepatocytes were isolated as in (12) and cell incubations were performed in rotating round-bottomed flasks (10^6 cells/ml) under an atmosphere of 95% O_2 5% CO_2. Glutathione reductase was inhibited by treatment of the cells with 1,3-bis-(2-chloroethyl)-1-nitrosourea essentially as previously described (13), except that Eagles minimal essential medium supplemented with 1 mM methionine was used for cell treatment and cell recovery. This treatment produces at least 90% inhibition of GSSG reductase without affecting cellular GSH content (13) and this was verified in these studies. Incubations were performed in Krebs-Henseleit buffer, pH 7.4, containing 12.5 mM Hepes at 37°C, and substrates were added in dimethylsulfoxide (0.4% of final volume except for toxicity curves of DMNQ and DENQ 0.6 mM (0.8%) and 1 mM (2%)). Control experiments showed that concentrations of up to 2% DMSO were not cytotoxic and had no effect on menadione-induced toxicity. Cell viability was determined by trypan blue exclusion. GSH and GSSG were determined by HPLC after de-

rivatization as described by Reed *et al.* (14). $\bar{O}_2^{\cdot}$ was detected by SOD-sensitive reduction of acetylated cytochrome *c*, prepared as in (15) using the wavelength pair 550–540 nm (16). Dialyzed whole liver homogenate was prepared by homogenization of the liver in 0.25 M sucrose (20% w/v), centrifugation at 2000*g* for 10 min, followed by overnight dialysis of the supernatant against 0.15 M KCl, pH 7.4 (21). Aliquots of supernatant (0.05 ml) were mixed with 0.15 M Tris–HCl buffer, pH 7.4 (1 ml), containing acetylated cytochrome *c* (1 mg/ml) and NADPH, 1 mM. In all experiments dicoumarol was used at a concentration of 30 μM and SOD at 0.1 mg/ml. SOD-sensitive reduction of ferricytochrome derivatives cannot be used as an absolute measure of $\bar{O}_2^{\cdot}$ production due to other competing reactions of $\bar{O}_2^{\cdot}$, such as enzymatic and nonenzymatic dismutation. Protein was determined according to the method of Lowry *et al.* (17).

RESULTS

Alkylation reactions of quinones generally proceed at the ortho position to the quinone moiety. Thus both the 2,3-dialkylated derivatives (DMNQ and DENQ) would not be expected to directly alkylate biological nucleophiles. This was verified experimentally using GSH as a model nucleophile. If conjugate formation between quinone and GSH did not occur then full recovery of thiol equivalents in terms of GSH remaining and GSSG formed should be possible. That this was the case during reaction of DMNQ and DENQ with GSH is shown in Table I.

The toxicity of menadione and its two alkylated analogs, DMNQ and DENQ, in isolated rat hepatocytes is shown in Fig. 1 (A–C). The results show that both of the substituted analogs were markedly less toxic than menadione. The lack of toxicity of DMNQ and DENQ could reflect their inability to alkylate cellular nucleophiles but may also be a consequence of diminished one-electron reduction by mitochondrial and microsomal reductases relative to menadione. This would in turn lead to a decreased generation of semiquinone radical, and active oxygen species such as $\bar{O}_2^{\cdot}$ and H_2O_2. Alternatively, the diminished toxicity of DMNQ and DENQ may be due to their higher affinity for the enzyme DT-diaphorase [NADPH (quinone acceptor) oxidoreductase], a two-electron reductase which catalyzes the reduction of quinones to hydroquinones without the generation of semiquinone radical intermediates (18). Thus, it has been proposed that this enzyme plays a protective role in quinone-induced toxicity (1, 19). A dialyzed whole liver homogenate, devoid of soluble cellular thiols (which may interact with quinones directly to generate $\bar{O}_2^{\cdot}$ (4, 20)), was used to investigate these possibilities. A decreased capacity of DMNQ and DENQ relative to menadione to generate species which caused acetylated cytochrome *c* reduction was evident using the dialyzed whole liver homogenate preparation (Fig. 2A). Reduction of the cytochrome can reflect generation of either hydroquinone[3] or $\bar{O}_2^{\cdot}$, as both

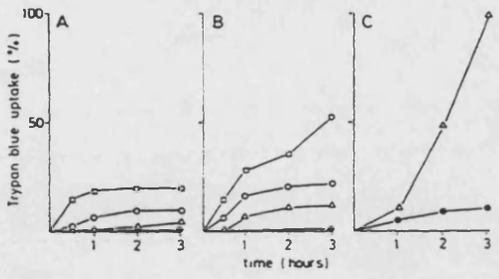

FIG. 1. Toxicity of 2,3-dimethyl-1,4-naphthoquinone (A), 2,3-diethyl-1,4-naphthoquinone (B), and menadione (C) in isolated hepatocytes. (Quinone concentrations were: ●, 0.1 mM; △, 0.2 mM; ○, 0.6 mM, □, 1 mM). One experiment typical of three.

TABLE I

RECOVERY OF THIOL EQUIVALENTS AFTER REACTION OF DMNQ OR DENQ (0.2 mM) WITH GSH (0.2 mM) FOR 30 min AT 37°C[a]

	GSH remaining (μM)	GSSG formed (μM GSH eq)	Total (GSH eq)
Without quinone	170	31	201
DMNQ	143	66	209
DENQ	138	66	204

[a] In Krebs-Henseleit buffer, pH = 7.4.

[3] We have found that the hydroquinone of menadione, synthesized according to L. Feiser [(1940) *J. Biol. Chem.* 133, 391–396], readily reduces acetylated cytochrome *c*.

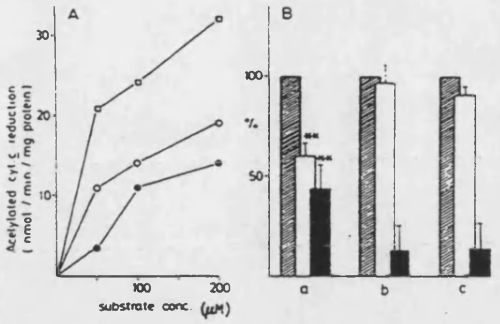

FIG. 2. NADPH-dependent reduction of acetylated cytochrome *c* induced by 1,4-naphthoquinones in dialyzed whole liver homogenate. (Menadione (□); 2,3-dimethyl-1,4-naphthoquinone (○); and 2,3-diethyl-1,4-naphthoquinone (●)). (A) Concentration dependence of NADPH-(1 mM) dependent acetylated cytochrome *c* reduction in dialyzed whole liver homogenate. (B) Effects of SOD (0.2 mg/ml, open bar), and dicoumarol (0.03 mM, solid bar) on NADPH-dependent reduction of acetylated cytochrome *c* in dialyzed whole liver homogenate. (a) Menadione, 0.2 mM; (b) 2,3-dimethyl-1,4-naphthoquinone, 0.2 mM; (c) 2,3-diethyl-1,4-naphthoquinone, 0.2 mM. Means ± standard deviation of three experiments.

are capable of transferring electrons to acetylated cytochrome *c*. (1). With menadione, however, 50% of the reduction was SOD sensitive, suggesting the involvement of $\bar{O}_2^{\cdot}$, whereas SOD had no significant effect on DMNQ- and DENQ-induced acetylated cytochrome *c* reduction (Fig. 2B). Conversely, dicoumarol, an inhibitor of DT-diaphorase (19), almost totally inhibited the NADPH-dependent reduction of the cytochrome by DMNQ and DENQ, whereas menadione-induced reduction was still 35% of the control value (Fig. 2B). These experiments demonstrate that the alkyl-substituted analogs of menadione have a higher ratio of two- as opposed to one-electron reduction relative to menadione itself. In agreement with previous studies (1, 21) and the protective role of DT-diaphorase, pretreatment of hepatocytes with dicoumarol (0.03 mM) markedly exacerbated the toxicity of all three quinones to hepatocytes (data not shown).

In an attempt to clarify the role of oxidative processes in naphthoquinone-induced toxicity we have used cells pretreated with BCNU, an inhibitor of glutathione reductase. This treatment exacerbates intracellular oxidative challenge, as any GSSG generated in the cell cannot be reduced back to GSH. Both DMNQ and DENQ caused some loss in hepatocyte GSH content (Figs. 3B-C) relative to controls (Fig. 3A). There was, however, a potentiation of GSH removal of GSSG generation in BCNU-pretreated cells relative to control cells in the case of all three naphthoquinones (Figs. 3A-D). Furthermore, the three naphthoquinones were significantly more toxic to BCNU-pretreated cells than to control cells (Figs. 4A-D), demonstrating that enhanced oxidative challenge can result in enhanced cellular toxicity.

DISCUSSION

In this study we have investigated the metabolism and toxicity of alkyl-substituted analogs of menadione which cannot alkylate cellular nucleophiles directly in order to determine the role of oxidative processes in cytotoxicity. The alkyl-substituted analogs of menadione, DMNQ and DENQ, were markedly less toxic to isolated hepatocytes than the parent compound. High concentrations of DMNQ and DENQ were required to induce significant cytotoxicity in hepatocytes. These data do not allow us to conclude, however, that oxidative processes are unimportant in menadione-induced cytotoxicity and that alkylation reactions represent the only determinants of the toxicity observed. An alternative explanation for the decreased toxicity of DMNQ and DENQ relative to menadione may be their low ability to generate oxidizing species within the cell—either because of low affinity for one-electron reductases or because of higher affinity for alternative pathways of metabolism such as DT-diaphorase-catalyzed two-electron reduction. Using dialyzed liver homogenate we found that SOD could substantially inhibit menadione-induced acetylated cytochrome *c* reduction, whereas it had minimal inhibitory effect on DMNQ- and DENQ-mediated reduction (Fig. 2). This suggests that $\bar{O}_2^{\cdot}$ generated by redox cycling reactions is responsible for a substantial proportion of the observed reduction of

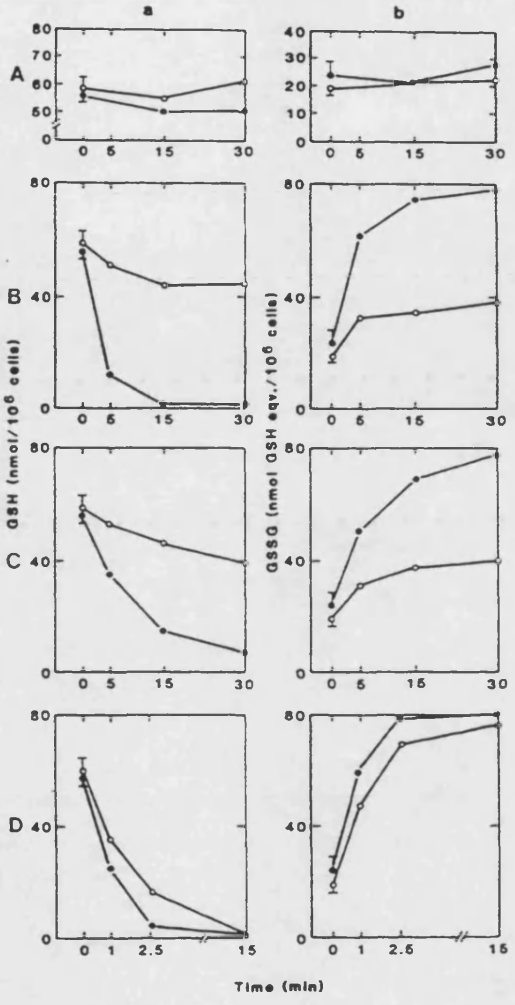

FIG. 3. GSH depletion (a) and GSSG generation (b) during metabolism of 2,3-dimethyl-1,4-naphthoquinone 0.2 mM (B), 2,3-diethyl-1,4-naphthoquinone 0.2 mM (C), and menadione 0.05 mM (D), in control (O) and BCNU-pretreated cells (●). The thiol status of control (O) and BCNU-pretreated (●) cells in the absence of quinone but in the presence of the solvent vehicle (DMSO) is shown in (A). One experiment typical of three.

acetylated cytochrome *c* only with menadione. Furthermore, data obtained using dicoumarol, an inhibitor of DT-diaphorase, showed that two-electron reduction was involved in the metabolism of all three naphthoquinones, but that this metabolic pathway was more active with DMNQ and DENQ. The alkylated analogs, therefore, undergo a higher ratio of two- as opposed to one-electron reduction relative to menadione and thus this represents a possible explanation of the decreased toxicity of DMNQ and DENQ relative to menadione.

Whether a particular quinone is metabolized by one- or two-electron reductases depends on a number of factors such as steric considerations, lipophilicity, and one-electron reduction potential. The dependence of the rate of $\bar{O}_2^{\cdot}$ production by isolated rat hepatocytes on the one-electron reduction potential of quinones has been studied previously by Powis *et al.* (22). These workers showed that in isolated hepatocytes maximal quinone-induced $\bar{O}_2^{\cdot}$ formation occurs at a quinone one-electron reduction potential of −70 mV (22). From their data one can also predict that a more negative one-electron reduction potential

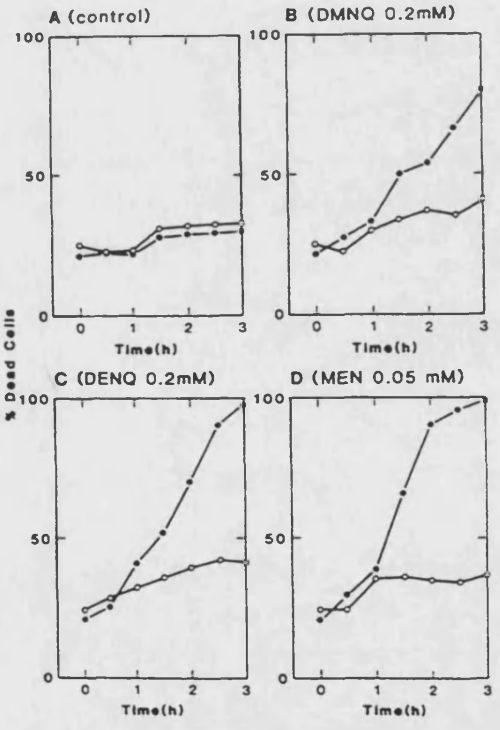

FIG. 4. Toxicity of 2,3-dimethyl-1,4-naphthoquinone 0.2 mM (B), 2,3-diethyl-1,4-naphthoquinone 0.2 mM (C), and menadione 0.05 mM (D), in control (O) and BCNU-pretreated cells (●). The toxicity of the solvent vehicle to control (O) and BCNU-pretreated (●) cells is shown in (A). One experiment typical of five.

than that of menadione (−200 mV) would lead to a marked decrease in the rate of superoxide formation. As alkyl substitution of the naphthoquinone nucleus would be expected to cause donation of electrons into the ring system, the one-electron reduction potentials of DMNQ and DENQ would be predicted to be more negative than menadione itself. Thus, on the basis of one-electron reduction potential alone, the alkyl-substituted derivatives of menadione would be expected to induce the generation of fewer oxygen-derived radicals.

The observation that DMNQ and DENQ were cytotoxic, albeit at high concentrations, suggests that oxidative processes alone can cause cytotoxicity. To investigate this we used cells preincubated with BCNU, which inhibits cellular glutathione reductase. In control cells, all three naphthoquinones induced some GSH loss and GSSG formation, but these changes were markedly exacerbated by BCNU pretreatment (Fig. 3), showing that the alterations in GSH homeostasis observed occur, at least in part, by means of oxidative mechanisms. Although DMNQ and DENQ did not induce significant formation of $\dot{O}_2^-$ in dialyzed liver homogenate (Fig. 2), naphthoquinones can still induce an oxidative challenge including $\dot{O}_2^-$ and H_2O_2 generation, via chemical reactions (23), and this may represent the route whereby DMNQ and DENQ induce oxidative stress.

As well as a potentiation of naphthoquinone-induced GSH loss and GSSG formation in BCNU-pretreated cells relative to control cells, marked potentiation of toxicity in the nitrosourea-treated cells was also observed. It seems probable that the potentiation of oxidative challenge observed in BCNU-pretreated cells contributes to the increased toxicity of the naphthoquinones, although our data is not conclusive proof of this. It is clear from the data shown in Figs. 3D and 4D that GSH loss alone does not necessarily result in cytotoxicity, thus confirming previous work (5).

When both alkylation and oxidation can play a role in quinone-induced cytotoxicity, for example in the case of 1,4-naphthoquinone (21) and menadione (5), the dissection of the relative importance of each process for the eventual cytotoxic effect observed is not a simple exercise. Indeed, as the alkylation of thiols by menadione (4, 20) and 1,4-naphthoquinone (G. Cohen, personal communication) produces active oxygen species, then the two processes are not mutually exclusive. In addition naphthoquinones may induce cytotoxicity by other undefined processes. Thus, it seems obvious that in the case of menadione and 1,4-naphthoquinone more than one mechanism contributes to cytotoxicity.

In this study we have shown that derivatives of menadione which cannot directly alkylate nucleophiles are still cytotoxic, albeit at high concentrations, in isolated hepatocytes. We have also investigated the toxicity of naphthoquinones in BCNU-pretreated cells. Increased quinone-induced changes in soluble thiol oxidation were observed in BCNU-pretreated cells and these changes were associated with exacerbated cytotoxicity. These data suggest that oxidative processes alone can function as a determinant of the cytotoxicity of naphthoquinones.

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PUBLICATION 21

Hepatotoxicity of *N*-Methylformamide in Mice. II. Covalent Binding of Metabolites of [^{14}C]-Labelled *N*-Methylformamide to Hepatic Proteins

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HEPATOTOXICITY OF N-METHYLFORMAMIDE IN MICE—II

COVALENT BINDING OF METABOLITES OF [^{14}C]-LABELLED *N*-METHYLFORMAMIDE TO HEPATIC PROTEINS

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Abstract—Incubation of the hepatotoxin *N*-methylformamide (NMF) labelled either in the methyl group ($OHCNH^{14}CH_3$) or the formyl group ($OH^{14}CNHCH_3$) with mouse hepatic microsomes in the presence of NADPH, but not in its absence, led to covalent binding of metabolites to microsomal proteins. When [^{14}C]NMF was injected into BALB/c mice radioactivity was found to be associated with liver and, to a much lesser extent, with kidney proteins. Association of radioactivity derived from $OHCNH^{14}CH_3$ with hepatic proteins was higher in BALB/c mice than in CBA/CA mice and in these it was higher than in BDF_1 mice. Association of label derived from either isotopomer was significantly reduced but not abolished by pretreatment of mice with cycloheximide suggesting both covalent binding and metabolic incorporation of NMF metabolites. Depletion of hepatic glutathione by pretreatment of mice with buthionine sulfoximine or diethyl maleate prior to administration of $OH^{14}CNHCH_3$ enhanced the association of label with hepatic proteins measured 1 hr after drug injection. Covalent binding of [^{14}C]NMF to hepatic microsomes *in vitro* was abolished in the presence of glutathione. It is argued that the generation of the toxic lesion and the association of NMF metabolites with hepatic proteins may be causally related even though certain mechanistic and enzymatic details of this link remain obscure.

The biotransformation of relatively inert chemicals to reactive metabolites, commonly referred to as "metabolic activation", is now considered to be an obligatory initial event in a number of toxicities induced by chemicals [1]. The experimental antitumour drug *N*-methylformamide (NMF) [2, 3] is known to be hepatotoxic [4, 5]. In an accompanying paper [6], compelling evidence is presented for the contention that NMF undergoes metabolic activation to a hepatotoxic metabolite. Reactive metabolites generated from such chemicals can bind covalently to cellular macromolecules, thus modifying their biological properties, possibly in a detrimental way [7]. The objectives of the work described in this paper were to provide a better understanding of the mechanism by which NMF causes hepatotoxicity, and, more specifically, to test the hypothesis that like other hepatotoxic drugs, NMF is metabolised to species which can bind covalently to hepatic macromolecules.

MATERIALS AND METHODS

Chemicals and animals. Materials were purchased in the purest commercially available forms: Glutathione, NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, phenobarbital and cycloheximide (Sigma Chemical Co., Poole, U.K.); diethyl maleate and NMF (Aldrich Chemical Co., Gillingham, U.K.). NMF was distilled before use. (±)-Buthionine sulfoximine was prepared as described previously [8] and [^{14}C]-labelled NMF with the label either in the methyl group, $OHCNH^{14}CH_3$, or in the formyl group, $OH^{14}CNHCH_3$, was also synthesized according to a published method [9].

Experiments were conducted using male BALB/c, CBA/CA or BDF_1 mice (18–25 g, supplier: Bantin and Kingman, Hull, U.K.). Animals were housed on wire mesh and allowed free access to tap water and food (Heygate 13 breeding diet, Pilsbury's Ltd., Birmingham, U.K.) in rooms with a daily light cycle from 6 a.m. to 6 p.m. All chemicals were injected i.p. after dissolution in isotonic saline (injection volume: 0.2 ml) except diethyl maleate which was injected as a solution in arachis oil (0.2 ml). Chemicals were administered at the following doses (unless otherwise stated) which were based on the references quoted: NMF, 400 mg/kg [3], cycloheximide, 2 mg/kg 45 min before NMF or leucine, and [^{3}H] leucine, 20 mg/kg with 2 μCi/mouse [10]; (±)-buthionine sulfoximine, 1600 mg/kg 4 hr before NMF [11]; diethyl maleate, 0.3 ml/kg 1 hr before NMF [12]. In the *in vivo* binding studies 5 μCi [^{14}C] NMF was administered together with the amount of NMF to make up the desired dose.

Determination of association of label with liver and kidney macromolecules in vivo. Tissues were excised, weighed and frozen until analysis. After thawing a 25% (w/v) homogenate in water was prepared. Proteins were precipitated with 3 vol. of acetone. The precipitate was exhaustively washed with water

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Table 1. Covalent binding of metabolites of [^{14}C]NMF to hepatic microsomal proteins

Experimental conditions*	Covalent binding (ng NMF equivalents/mg protein, mean ± SD) OHCNH^{14}CH$_3$	OH^{14}CNHCH$_3$
Microsomes	14.6 ± 2.0 (7)†	14.4 ± 6.1 (4)
Heat-inactivated microsomes with NADPH	32.4 ± 5.1 (3)	13.8 ± 2.6 (3)
Microsomes with NADPH	123.6 ± 13.2 (9)‡	60.7 ± 9.8 (5)‡
Microsomes, NADPH and glutathione (10 mM)	10.0 ± 3.5 (3)§	13.9 ± 6.2 (3)‖

* [^{14}C]NMF (7 mM) was incubated with microsomes obtained from BALB/c mice for 2 hr as described under Materials and Methods.
† Number of experiments in brackets.
‡ Significantly different from microsomes alone ($P < 0.001$).
§‖ Significantly different from microsomes with NADPH (§$P < 0.001$, ‖$P < 0.01$).

(10 times) and water : methanol (1 : 1) (5 times), after which no further non-covalently bound radioactivity could be removed. The tissue pellet was solubilized in 2 ml of soluene 350 (Packard United Technologies, Reading, U.K.). The amount of protein which had been precipitated was determined according to Lowry *et al.* [13], protein loss during the washing procedure was less than 5%. The results were calculated as ng NMF equivalents bound to 1 mg protein, unless described otherwise, and are either presented as such or as percent of control binding. Radioactivity bound to DNA and RNA was determined as summarized by Pohl and Branchflower [14].

Determination of covalent binding to metabolites of [^{14}C]*NMF to liver microsomes* in vitro. Livers were excised from BALB/c mice at 9 a.m. and a homogenate (20% w/v) was prepared in phosphate buffer (50 mM, pH 7.4). Microsomes were prepared in the standard way involving centrifugation of the homogenate at 9000 *g* for 20 min and centrifugation of the 9000 *g* supernatant at 100,000 *g* for 60 min. The microsomal pellet was resuspended in phosphate buffer and aliquots (equivalent to 200 mg liver) were incubated in open beakers with [^{14}C]NMF (7 mM, specific activity 71 μCi/mol). This concentration equals the peak concentration of NMF in the blood of mice which received 400 mg/kg NMF [15]. The final incubation volume measured 2 ml and, when appropriate, contained also NADP (1 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (2 units/ml) as NADPH generating system and $MgCl_2$ (5 mM). The metabolic viability of the microsomes was assessed in control incubations with aminopyrine. Aminopyrine metabolism to formaldehyde was determined according to Nash [16]. Under the conditions of the covalent binding assay aminopyrine (5 mM) was metabolized to 5.5 ± 0.8 nmoles HCHO/mg protein/min (mean ± SD, N = 3) in the presence of NADPH and to 0.9 ± 0.2 nmoles HCHO/mg protein/min without NADPH. Covalent binding experiments were carried out in duplicate. Incubations were performed with shaking at 37° for 2 hr. Microsomal proteins were precipitated with acetone (4 ml). The precipitate was washed as described for the *in vivo* experiments and the pellet obtained was dissolved in 1 M NaOH (2 ml). Aliquots of the solute were used for protein determination (50 μl) and for scintillation counting (1 ml).

Measurement of radioactivity Aqueous samples (1 ml) were mixed with Fisofluor mpc scintillant (10 ml, Fisons Ltd., Loughborough, U.K.), tissue protein samples dissolved in soluene 350 (1 ml) were mixed with Beckman Readysolve (10 ml; Beckman Instruments Ltd., High Wycombe, U.K.) and the dissolved microsomal proteins in aqueous NaOH (1 ml) were mixed with Dimilume 30 (10 ml; Packard United Technologies). Radioactivity was counted in a Packard Tricarb 2660 scintillation counter using the external standardization mode. For all samples the counting efficiency was > 80%.

Statistical analysis. Results were compared using Student's *t*-test.

RESULTS

On incubation with hepatic microsomes and an NADPH generating system, [^{14}C]NMF was metabolised to compounds which bound covalently to microsomal protein (Table 1). Approximately twice as much radioactivity derived from OHCNH^{14}CH$_3$ was bound than activity derived from OH^{14}CNHCH$_3$. When [^{14}C]NMF was administered to BALB/c mice it bound to hepatic proteins in a dose dependent manner (Fig. 1). Injection of 20 mg/kg led to only negligible binding. At the dose of 400 mg/kg approximately twice as much radioactivity was associated with proteins when OHCNH^{14}CH$_3$ (Fig. 1A) was injected as compared to OH^{14}CNHCH$_3$ (Fig. 1B) which parallels the results obtained in the *in vitro* experiments (Table 1). When calculated as percentage of the 400 mg/kg dose, 2.4 ± 0.2% in the case of OHCNH^{14}CH$_3$ and 0.8 ± 0.3% in the case of OH^{14}CNHCH$_3$ were found bound to liver macromolecules 8 hr after drug administration. Activity was not associated with nucleic acids. Therefore, we assume that metabolites were associated mainly, if not exclusively, with proteins.

In the kidneys, an organ to which NMF does not appear to be toxic, label derived from OHCNH^{14}CH$_3$ was also bound to a much greater extent than label

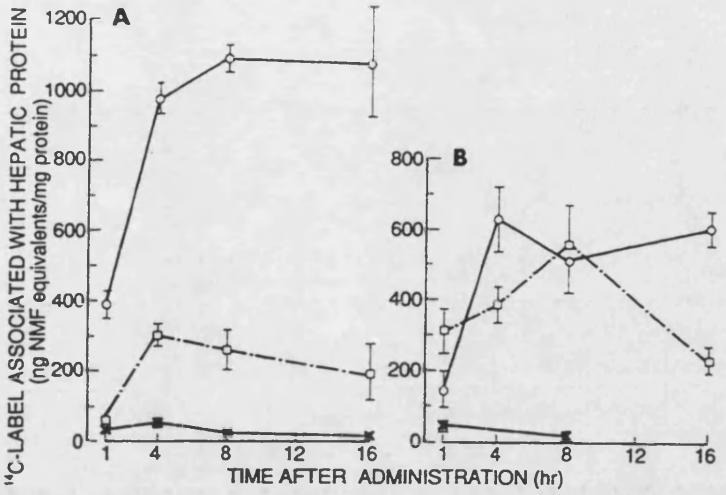

Fig. 1. Relationship to dose of the association of label derived from $OHCNH^{14}CH_3$ (A) and $OH^{14}CNHCH_3$ (B) with hepatic proteins in BALB/c mice; ×– – –× 20 mg/kg, □–·–·–□ 100 mg/kg, ○——○ 400 mg/kg. Association of activity with proteins was measured as described under Material and Methods. Values are the mean ± SD of 3 mice.

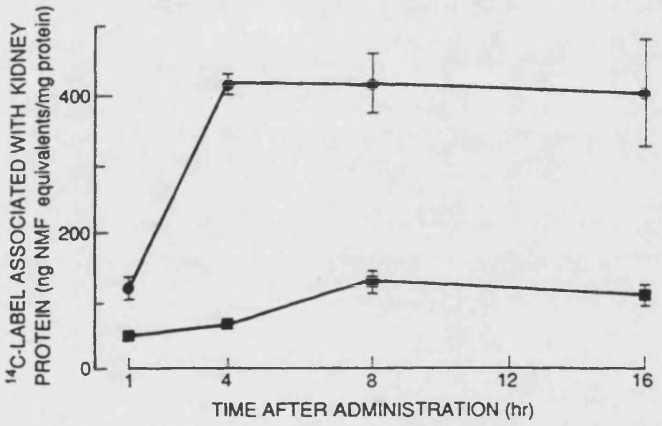

Fig. 2. Association of label derived from 400 mg/kg $OHCNH^{14}CH_3$ (●) or $OH^{14}CNHCH_3$ (■) with kidney proteins in BALB/c mice. Activity associated with proteins was determined as described under Material and Methods. Values are the mean ± SD of 3 mice.

derived from $OH^{14}CNHCH_3$ (Fig. 2). The radioactivity found in the kidneys 8 hr after administration of 400 mg/kg [^{14}C]NMF was only 36% of that associated with liver proteins in the case of $OHCNH^{14}CH_3$ and 25% after injection of $OH^{14}CNHCH_3$. Radioactivity associated with other tissues (e.g. lungs, spleen, heart) was less than a quarter of that measured in the liver (results not shown). In these tissues NMF does not appear to cause toxicity [17].

There was a marked difference between strains of mouse in the extent to which metabolites of [^{14}C]NMF were bound to or incorporated into proteins. Figure 3 shows that association of radioactivity was highest in BALB/c mice, both in the liver and the kidneys. In the case of $OHCNH^{14}CH_3$, the amount of label associated with hepatic proteins in CBA/CA mice was significantly lower than that measured in BALB/c mice but significantly higher than that measured in BDF_1 mice (Fig. 3).

In order to distinguish between covalent binding and incorporation of [^{14}C]NMF metabolites, bound radioactivity was determined in livers of mice which had been pretreated with cycloheximide, an inhibitor of protein synthesis, prior to drug administration. The decrease in association of label from [^{14}C]NMF with liver proteins caused by cycloheximide was greater than (1 hr after NMF injection) or similar to (8 hr after NMF administration) the effect which cycloheximide exerted on the incorporation of [^{14}C] leucine (Fig. 4), indicating that metabolites of NMF were incorporated to some extent into proteins via precursors of endogenous substrates. However, this result has to be interpreted with caution as the possibility cannot be excluded that cycloheximide affects the enzymes involved in the metabolism of NMF to the reactive species.

With the view of studying the role of glutathione in the processes leading to the association of NMF

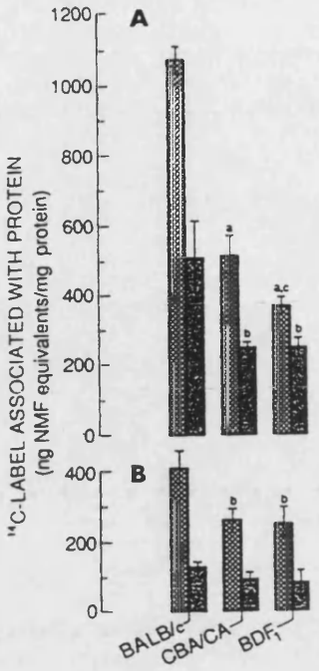

Fig. 3. Association of label derived from 400 mg/kg $OHCNH^{14}CH_3$ (hatched bars) or $OH^{14}CNHCH_3$ (stippled bars) with proteins in the liver (A) and the kidneys (B) of BALB/c, CBA/CA and BDF_1 mice. Values are the mean ± SD of 3 mice; [a,b]significant difference compared to BALB/c mice ([a]$P < 0.001$, [b]$P < 0.01$), [c] significant difference compared to CBA/CA mice ($P < 0.05$).

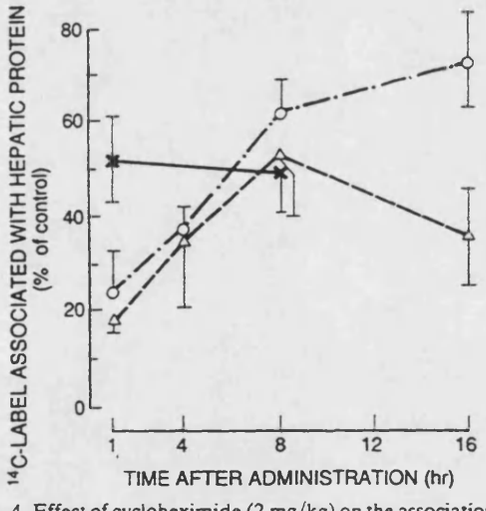

Fig. 4. Effect of cycloheximide (2 mg/kg) on the association of label derived from $OHCNH^{14}CH_3$ (O–·–·–O) or $OH^{14}CNHCH_3$ (Δ– – –Δ) with hepatic proteins, or on the incorporation of [^{3}H] leucine (×——×) into hepatic proteins in BALB/c mice. Cycloheximide (2 mg/kg) was administered 45 min prior to either 400 mg/kg [14C]NMF or 2 μCi [^{3}H]leucine. Results are expressed as percentage of values measured in mice which were not pretreated with cycloheximide (controls), on the basis of ng NMF equivalents per mg protein. Values are the mean ± SD of 3 mice.

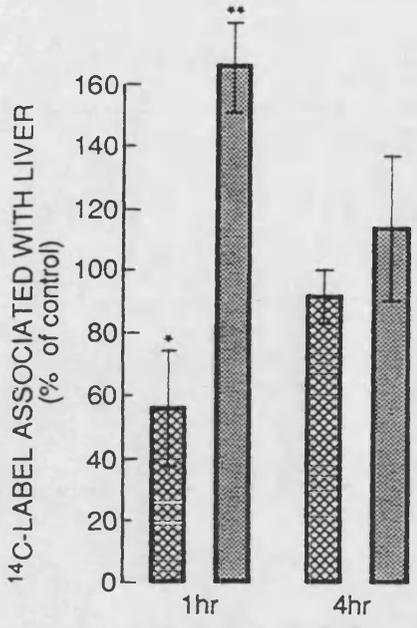

Fig. 5. Influence of pretreatment with buthionine sulfoximine (1600 mg/kg) on the association of label derived from $OHCNH^{14}CH_3$ (hatched bars) or $OH^{14}CNHCH_3$ (stippled bars) with hepatic proteins in BALB/c mice. Buthionine sulfoximine (1600 mg/kg) was injected 4 hr prior to [^{14}C]NMF (400 mg/kg) and label was measured 1 hr and 4 hr after NMF administration. Association is expressed as percent of binding observed in animals which were not pretreated with buthionine sulfoximine (controls), calculated on the basis of the amount of metabolites associated with the whole liver rather than with a mg of protein. As in these animals necrosis occurred and blood proteins accumulated in the livers within 4 hr of NMF administration, this representation was considered to reflect changes in association appropriately [20]. Values are the mean ± SD of 3 mice; stars indicate significant difference with controls, *$P < 0.05$, **$P < 0.005$.

metabolites with hepatic proteins, hepatic glutathione was depleted by administration of buthionine sulfoximine, an inhibitor of glutathione synthesis, prior to administration of [^{14}C]NMF. This pretreatment led to a decrease in hepatic glutathione to 19% of control levels at the time of [^{14}C]NMF injection [6]. Whereas pretreatment with buthionine sulfoximine inhibited the association of label derived from $OHCNH^{14}CH_3$ to a moderate extent, it markedly enhanced the association in the case of $OH^{14}CNHCH_3$ when covalently bound label was determined 1 hr after NMF administration (Fig. 5). However, 4 hr after administration, radioactivity derived from either radioisotopomer was associated with liver proteins to an extent similar to that observed in control mice. At this time livers of mice which had received NMF after buthionine sulfoximine exhibited severe haemorrhagic lesions and some animals were clearly moribund. Therefore, the interpretation of the amount of radioactivity associated with hepatic proteins at this stage may not be meaningful. Nevertheless, such hepatic damage was not apparent on macroscopic inspection of livers

1 hr after administration of NMF. Pretreatment of mice with diethyl maleate, a compound which depletes hepatic glutathione by avid electrophilic attack, also raised the level of activity associated with proteins 1 hr after administration of $OH^{14}CNHCH_3$ to 218 ± 20% (mean ± SD, N = 3) of control, whereas the association of label derived from $OHCNH^{14}CH_3$ was only 33 ± 9% (N = 3) of that observed in control mice. Thus, removal of hepatic glutathione appears to increase the propensity of liver proteins towards association with metabolites of the formyl moiety of the NMF molecule, a finding congruent with the observation that the covalent binding from both isotopomers of [^{14}C]NMF to hepatic microsomes *in vitro* was completely abolished in the presence of 10 mM glutathione (Table 1).

DISCUSSION

It is shown here for the first time that metabolites of NMF bind covalently to hepatic macromolecules, and there are three pieces of evidence which suggest that the generation of the toxic lesion caused by NMF and the covalent binding of it metabolites are causally related.

(i) The binding observed *in vivo* was appreciably higher in the organ in which toxicity is expressed than in other tissues.

(ii) The rank order of association of metabolites of [^{14}C]NMF with liver macromolecules in three strains of mouse *in vivo* (Fig. 3) paralleled differences between the sensitivity of these strains towards NMF-induced hepatotoxicity and hepatic glutathione depletion [6].

(iii) Modulation of the hepatic glutathione status prior to NMF administration affected both the severity of the hepatic damage and the binding in a fashion which suggests the involvement of a reactive, toxic intermediate. Depletion of hepatic glutathione exacerbated toxicity and increased binding of metabolites of the NMF formyl moiety *in vivo* (Fig. 5). Conversely, pretreatment of mice with thiol compounds protected the livers against toxicity and, likewise, the presence of glutathione in the incubation medium totally abolished covalent binding measured *in vitro* (Table 1).

The radioactivity found to be associated with the hepatic proteins *in vivo* was at least partially due to incorporation into proteins of metabolites which were precursors of endogenous substrates. This conclusion is based on the finding that inhibition of protein synthesis markedly decreased the association. What percentage of the overall association was due to covalent adduct formation is difficult to establish. However, since covalent binding occurred *in vitro*, it is likely that it also contributed to overall association with tissue macromolecules *in vivo*.

There was a difference between the two [^{14}C]NMF isotopomers with regard to both the extent of binding *in vitro* and *in vivo* and the relationship between dose and their binding *in vivo*. This suggests that metabolic rupture of the amide bond in the NMF molecule precedes binding or incorporation of at least a portion of the dose of [^{14}C]NMF injected. The end products of NMF metabolism have recently been identified [18]. Carbon dioxide and methylamine are the major metabolites of NMF and both, or their immediate precursors, could conceivably contribute to incorporation of label into protein, as they are substrates of endogenous metabolic pathways. On the basis of the recent finding that *S*-(*N*-methylcarbamoyl)-*N*-acetylcysteine is a urinary metabolite of NMF in rodents and man [19], a number of chemically plausible electrophilic metabolic intermediates of NMF can be proposed. The formation of these intermediates could explain both the hepatic necrosis [4, 5] and the hepatic covalent binding which NMF causes in mice. Which enzyme system is responsible for the activation of NMF is still unclear.

Pretreatment of mice with buthionine sulfoximine exacerbated NMF toxicity [6] and it increased the association of label derived from $OH^{14}CNHCH_3$ with liver proteins *in vivo*. Assuming that the binding characterised in this study is indeed linked to the genesis of the toxic lesion one could conclude that the metabolic fate of the NMF formyl moiety is more crucial for the development of toxicity than that of the methyl moiety. It is, however, difficult to reconcile this conclusion with the difference between the two isotopomers in the relationship of the binding to dose. The amount of radioactivity derived from $OH^{14}CNHCH_3$ which was found in the liver after administration of the toxic dose of 400 mg/kg was only approximately twice the amount bound after the innocuous dose of 100 mg/kg, whereas in the case of $OHCNH^{14}CH_3$ the level of association after 400 mg/kg was approximately four times that observed after 100 mg/kg (Fig. 1). This would indicate that the extent of binding of the $^{14}CH_3$-labelled isotopomer reflects more convincingly the dose threshold for toxicity than does the binding of the ^{14}CHO-labelled isotopomer. This interpretation would be in accordance with the postulate that events leading to the association of metabolites of the NMF formyl moiety are less important for toxicity than those causing the binding of metabolites of the methyl group.

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PUBLICATION 22

An Investigation of the Relationship between the Hepatotoxicity and the Metabolism of *N*-Alkylformamides

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An Investigation of the Relationship between the Hepatotoxicity and the Metabolism of N-Alkylformamides[1]

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ABSTRACT

The hepatotoxicity and metabolism of the following close analogs of the hepatotoxic antitumor agent N-methylformamide (NMF) were investigated in CBA/CA mice: N-ethylformamide (NEF), dimethylformamide (DMF), formamide and N-methylacetamide (NMA). Apart from NMF only NEF was potently hepatotoxic as measured by the elevation of plasma activities of the enzymes sorbitol dehydrogenase and alanine and aspartate aminotransferases 24 hr after drug administration. In freeze-dried urine samples of mice which had received NEF or NMF, but not in the case of DMF, formamide or NMA, thioesters were detected by thin-layer chromatography. Evidence based on high-pressure liquid chromatography analysis and 400 MHz ^{1}H-NMR and mass spectrometry suggests that the thioester metabolite of NEF is *S*-(N-ethylcarbamoyl)-N-acetylcysteine. It has been shown previously that NMF is metabolized to *S*-(N-methylcarbamoyl)-N-acetylcysteine. NEF also underwent extensive metabolism to ethylamine; similarly NMF was biotransformed to methylamine. In contrast, the urine of mice which had received DMF contained only very small amounts of dimethylamine and methylamine could not be detected as a metabolite of NMA. Instead, the major metabolite of NMA was identified by 400 MHz ^{1}H-NMR spectrometry as N-(hydroxymethyl)acetamide. DMF is known to undergo extensive metabolism to its N-hydroxymethyl derivative. The results suggest that two metabolic pathways of N-alkylformamides can be distinguished: Hydroxylation at the α-carbon of the N-alkyl group and oxidation of the formyl moiety. The former pathway presumably constitutes a detoxification route, and the latter may well be associated with hepatotoxicity, and affords a glutathione conjugate, excreted in the urine as a mercapturate.

N-Alkylformamides are important industrial chemicals as starting materials for chemical syntheses and as solvents. Their solvent properties are related to their high dipole moment and to their extensive miscibility with both aqueous and organic media. Among the N-alkylformamides, DMF is the compound used most frequently as a solvent. It has been found to be hepatotoxic in rodents (Dexter *et al.*, 1982; Lundberg *et al.*, 1981; Massmann, 1956; Mathew *et al.*, 1980) and in workers after occupational exposure to high concentrations of the vapor (Chivers, 1978; Finzel, 1972; Potter, 1973; Reinl and Urban, 1965; Tolot *et al.*, 1969; von Klavis, 1970). NMF shows antineoplastic activity in mice (Clarke *et al.*, 1953, Furst *et al.*, 1955; Gescher *et al.*, 1982; Gate *et al.*, 1986) and its preliminary clinical investigation as an antitumor drug (Laird Myers *et al.*, 1956; McVie *et al.*, 1984; Ettinger *et al.*, 1985) as well as experiments in mice (Langdon *et al.*, 1985; Whitby *et al.*, 1984) have shown it to be a hepatoxin. The mechanisms by which DMF and NMF cause toxicity are poorly understood. NMF has been proposed as proximate or ultimate toxic metabolite through which DMF exerts its hepatotoxicity (Lundberg *et al.*, 1981). However, this is unlikely, as it has been demonstrated recently that NMF is not a major urinary metabolite of DMF in rodents (Brindley *et al.*, 1983; Scailteur and Lauwerys, 1984; Kestell *et al.*, 1986a). As far as the mechanism of toxicity of NMF is concerned, evidence has emanated from our laboratories which suggests that NMF is metabolized to a chemically reactive, potentially toxic, compound which is able to bind covalently to hepatic macromolecules (Pearson *et al.*, 1986a) and causes the depletion of hepatic glutathione stores (Gescher *et al.*, 1982). The major urinary metabolites of NMF are methylamine (Kestell *et al.*, 1985) and *S*-(N-methylcarbamoyl)-N-acetylcysteine (Kestell *et al.*, 1986b). The identification of the latter metabolite has prompted the postulate that its formation, which involves oxidation at the formyl carbon, is a biotransfor-

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ABBREVIATIONS: DMF, N,N-dimethylformamide; NMF, N-methylformamide; NEF, N-ethylformamide; F, formamide; NMA, N-methylacetamide; SDH, sorbitol dehydrogenase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography; GLC, gas-liquid chromatography.

mation route which leads *via* the hepatotoxic intermediate. In the present paper, this hypothesis is being tested further. The hepatotoxic potential in the mouse of four close analogs of NMF has been investigated, namely of DMF, NEF, F and NMA (for structures see table 1). The hepatotoxic potencies of these compounds are compared with their proclivity to undergo oxidative and conjugative metabolism giving *S*-(N-carbamoyl)mercapturates *via* a pathway analogous to that which affords *S*-(N-methylcarbamoyl)-N-acetylcysteine in case of NMF (Kestell *et al.*, 1986b). The results presented in this paper demonstrate that, among the amides examined, only NEF and NMF are hepatotoxins in the mouse and also only NEF and NMF are metabolized to thiocarbamates.

Materials and Methods

The amides NMF, NEF, DMF, F and NMA were purchased from Aldrich Chemical Co. (Poole, U.K.). *S*-(N-methylcarbamoyl)-N-acetylcysteine was prepared as described by Kestell *et al.* (1986b) and the synthesis of the following thiocarbamates will be reported elsewhere: *S*-(N-ethylcarbamoyl)-N-acetylcysteine, *S*-(N,N-dimethylcarbamoyl)-N-acetylcysteine and *S*-(carbamoyl)-N-acetylcysteine. N-(Hydroxymethyl)acetamide was made as described by Chwala (1948) and [^{14}C] methylene-labeled NEF ($OHCNH^{14}CH_2CH_3$) was synthesized according to a method published previously for [^{14}C]methyl-labeled NMF ($OHCNH^{14}CH_3$) (Threadgill and Gate, 1983).

Amides were dissolved in saline solution and administered *via* the i.p. route to male CBA/CA mice (18–25 g). The injection volume was 0.2 ml and, in the metabolism experiments, the dose was 400 mg/kg.

In order to measure the extent of hepatotoxicity, the activities in plasma of the following three enzymes were assayed 24 hr after drug administration. SDH, as described by Rose and Henderson (1975), and AST and ALT as described by Kachmar and Moss (1976).

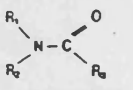
For the detection of *S*-(N-alkylcarbamoyl)-N-acetylcysteines as metabolites of formamides, urine samples were freeze-dried after acidification with 5 M HCl to achieve pH 1. In acidic media these *S*-(N-carbamoyl)-mercapturates are of sufficient stability to allow detection. The residues were suspended in methanol and analyzed both by HPLC and TLC. For the HPLC analysis, a Waters trimodular system (Waters Associates, Northwich, U.K.) fitted with a Waters RCM-100 radial compression unit and a C_{18} 5 μm reverse phase column was used, UV detection was achieved with a Waters λ_{max}480 LC spectrophotometer set at 205 nm. The eluant (0.01 M octylamine hydrochloride in water, pH 6.0: methanol, 3:1 v/v) was pumped through the column at a flow rate of 1 ml/min. TLC analysis was conducted on silica gel 60-coated plates (0.2 mm thickness, Merck, A.G., Darmstadt, W. Germany). Plates were developed in butan-1-ol-water-methanol (8:2:1 v/v). The mercapturates were detected by spraying the plates with either 1 M aqueous NaOH followed by Ellman's reagent (Glaser *et al.*, 1970) or with chloroplatinate reagent (Barnsley *et al.*, 1964). *S*-(N-Ethylcarbamoyl)-N-acetylcysteine was identified as an NEF metabolite after isolation by preparative TLC and esterification with methanolic hydrogen chloride as described previously for *S*-(N-methylcarbamoyl)-N-acetylcysteine (Kestell *et al.*, 1986b).

^{1}H-NMR and mass spectra of the methyl esters of metabolite and authentic thiocarbamate were obtained, respectively, at 400 MHz using a Bruker WH 400 spectrometer with D_2O as solvent, and using a VG 7070 mass spectrometer (with a VG 2035 data system) in the chemical ionization mode, using 2-methylpropane as reagent gas, run at a scan rate of 1 sec/decade. 400 MHz ^{1}H-NMR spectra of urine samples involved suppression of the H_2O signal by selective presaturation (1.5 sec) followed by collection of free induction decay, with 4 preliminary dummy scans. Unchanged amides in the urine were quantitated by GLC as described previously by Gescher *et al.* (1982). N-(hydroxymethyl)-acetamide was identified as a metabolite of NMA by 400 MHz ^{1}H-NMR analysis after separation of the constituents of freeze-dried urine samples by preparative TLC using benzene-acetone-acetic acid (3:2:2 v/v) as solvent and phenylhydrazine-ferric chloride reagent [prepared according to Nair and Francis (1980)] as detecting spray. After elution of the material from the TLC plate residual acetic acid and water were removed by freeze drying. N-(hydroxymethyl)acetamide was quantitated by GLC analysis under conditions used for the measurement of NMA. On the GLC column, this carbinolamide decomposes quantitatively to acetamide. Quantitative analysis was feasible as ^{1}H-NMR spectra of urine obtained after NMA administration showed that there was no appreciable excretion of acetamide generated metabolically from NMA.

Aliphatic amines in the urine were detected by HPLC after derivatization with dinitrobenzenesulfonic acid and extraction into ether as described previously (Kestell *et al.*, 1985). Methylamine and ethylamine were quantitated by counting of radioactivity after TLC separation (Kestell *et al.*, 1985) of urinary constituents obtained from mice which had received $OHCNH^{14}CH_3$ or $OHCNH^{14}CH_2CH_3$.

TABLE 1

Structures of amides used in this study



Amide	R_1	R_2	R_3
F	H	H	H
NMF	CH_3	H	H
NEF	C_2H_5	H	H
DMF	CH_3	CH_3	H
NMA	CH_3	H	CH_3

Results

In order to investigate the hepatotoxic potential of four close analogs of NMF, different doses of these amides were administered to mice and, after 24 hr, the plasma activities of three marker enzymes of hepatic damage, SDH, AST and ALT, were measured. In accordance with observations published previously (Langdon *et al.*, 1985), NMF was hepatotoxic at doses exceeding 200 mg/kg (3.4 mmol/kg) (fig. 1). Likewise, NEF exerted marked liver toxicity at 600 mg/kg (8.2 mmol/kg). However, none of the other three amides at doses of up to 3 g/kg caused plasma activities of the marker enzymes to increase as dramatically as seen after NMF. Nevertheless, even though F, DMF and NMA exhibited low hepatotoxic potential, at this dose they caused the mice to lose weight (table 2).

To study the formation of *S*-(N-alkylcarbamoyl)-mercapturates as urinary metabolites of the amides under test, freeze-dried urine samples from mice which had received either NMF, NEF, F or DMF were analyzed by TLC. The appropriate authentic mercapturates were prepared as reference compounds. Only the two monoalkylformamides NMF and NEF, but not DMF or F, gave rise to the appearance of thiocarbamates in the urine, as shown by TLC and HPLC analysis. The mercapturates arising from metabolism of NMF and NEF had R_fs identical to those of the respective synthetic materials, *S*-(N-methylcarbamoyl)-N-acetylcysteine (0.30) and *S*-(N-ethylcarbamoyl)-N-acetylcysteine (0.37). Both metabolites gave a positive reaction with Ellman's reagent after treatment with aqueous base as described under "Materials and Methods," indicating the presence of thioesters/thiocarbamates. The limit of detection by HPLC for unlabeled *S*-(N-alkylcarbamoyl)-mercapturates in the urine was near 10 μmol/ml. Therefore we cannot exclude the possibility that small amounts of DMF or

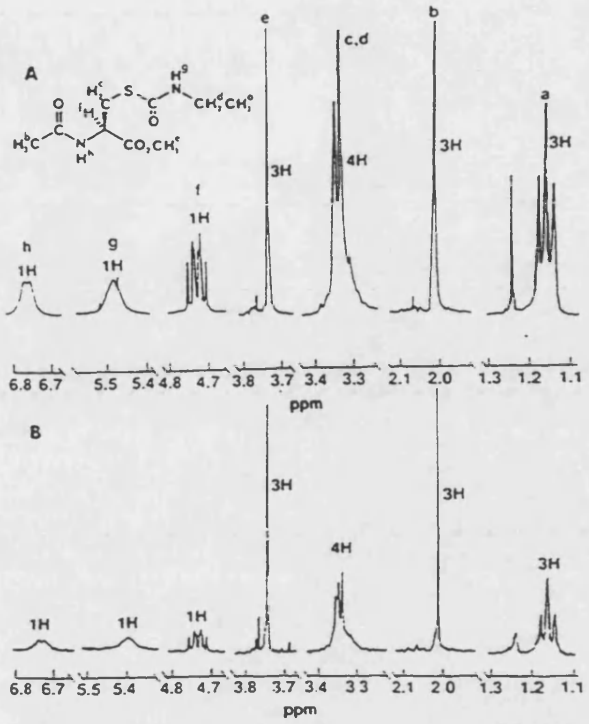


Fig. 3. Prominent signals in the 400 MHz ^{1}H-NMR spectra of *S*-(N-ethylcarbamoyl)-N-acetylcysteine methyl ester (A) and of a urinary metabolite of NEF after reaction with methanolic hydrogen chloride (B). Details of the metabolite isolation are described under "Materials and Methods."

TABLE 3

Amounts of unchanged amides and of metabolically generated alkylamines excreted in the urine of mice which received NMF, NEF, DMF or NMA (400 mg/kg)

Amide Administered	Amount Excreted Unchanged (% of dose ± S.D.)	Amine Detected[a]	Amount Excreted as Amine (% of dose ± S.D.)
NMF	26.4 ± 1.9 (5)[b]	CH_3NH_2	15.0 ± 1.9 (3)
NEF	4.8 ± 1.9 (5)	$C_2H_5NH_2$	42.1 ± 2.0 (4)
DMF	4.9 ± 1.4 (5)[c]	$HN(CH_3)_2$	3.9 ± 1.9[d]
		CH_3NH_2	4.1 ± 0.8[d]
NMA	2.0 ± 2.0 (6)	N.D.[e]	

[a] Urine was collected for 24 hr and amides and amines were detected and quantitated as described under "Materials and Methds."
[b] Numbers in parentheses, number of animals.
[c] As reported by Brindley *et al.* (1983).
[d] As reported by Kestell *et al.* (1986a).
[e] N.D., methylamine not detected.

spectrum of urine samples of mice which had received NMA is dominated by the acetyl proton resonance frequency of its major urinary metabolite (2.01 ppm). This metabolite was separated from other urine constituents by TLC. Figure 4B shows its ^{1}H-NMR spectrum which is a virtually identical with that of synthetic N-hydroxymethylacetamide (fig. 4A). Whereas 54 ± 10% (mean ± S.D., $n = 5$) of the dose of NMA was metabolized to N-hydroxymethylacetamide, only 2% was excreted as unchanged NMA (table 3).

Discussion

Among the formamides, NMF is the most effective antineoplastic agent in mice (Gate *et al.*, 1986). According to the results

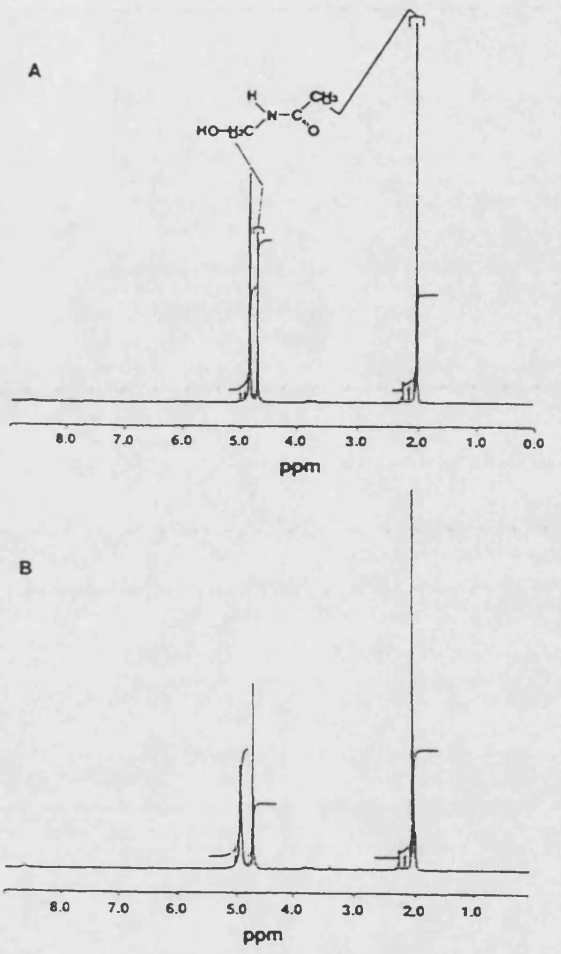


Fig. 4. ^{1}H-NMR spectra of N-hydroxymethylacetamide (A) and the major urinary metabolite of NMA (B) in D_2O. Details of the synthesis of N-(hydroxymethyl)acetamide and of the metabolite isolation are described under "Materials and Methods."

presented in this paper, the monoalkylformamides NMF and NEF appear also to be more potently hepatotoxic than other formamides. The amides investigated here seem to exert toxicities also in targets other than the liver, as the derivatives which lacked marked hepatotoxic potential caused marked weight loss at high doses (3 g/kg).

Little is known about the mechanism by which NMF causes toxicity. Studies of the effect of NMF on glutathione status and of the effects of manipulation of glutathione status on hepatotoxicity and covalent binding of metabolic products of radiolabeled NMF suggest that NMF undergoes metabolic activation to a reactive, potentially toxic, metabolite (Whitby *et al.*, 1984; Pearson *et al.*, 1987a,b). The analytical chemical work described here demonstrates that the two hepatotoxins among the five amides tested in this study are also the ones which underwent metabolism to *S*-(N-alkylcarbamoyl)-mercapturates. This result is good evidence for the contention that the biotransformation route (fig. 5A) which yields thiocarbamates is linked closely with the formamide-induced generation of the hepatotoxic lesion. The formation of the N-alkylthiocarba-

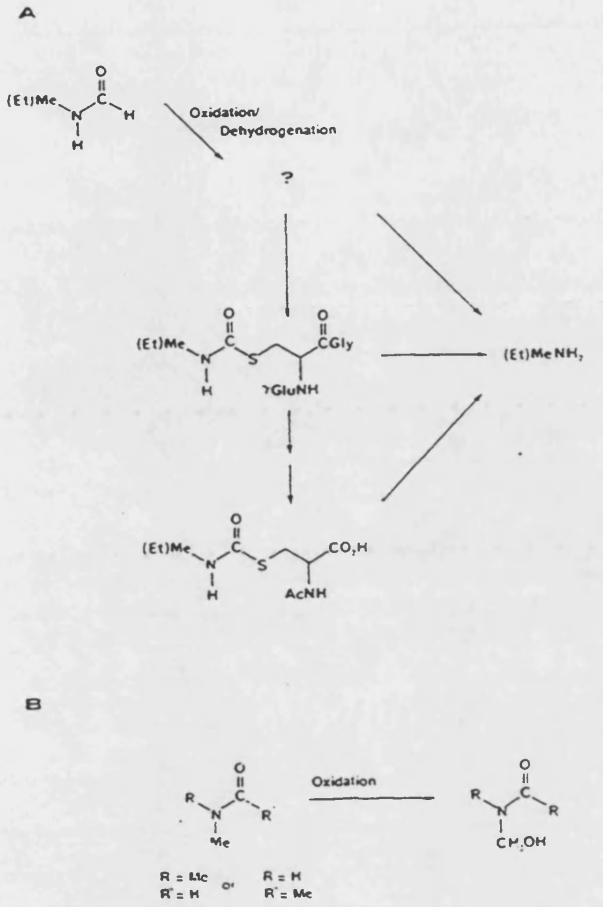


Fig. 5. Major metabolic toxification (A) and detoxification (B) pathways of N-alkylformamides and NMA.

mates from N-alkylformamides involves the oxidation of the formyl moiety and one could postulate a number of conceivable metabolic precursors, among them alkylisocyanates (alkyl-N=C=O). The formation of such a powerful electrophile would explain the fulminant hepatic necrosis observed in mice which had received NMF (Whitby *et al.*, 1984). It would appear (fig. 1) that NMF is slightly more toxic, on a molar basis, than is NEF and that this difference might correlate with the greater amount of mercapturic acid found in the urine, although this association probably does not indicate causality. It is worth noting that NMF and NEF are also the derivatives among the amides which gave rise to large amounts of metabolically produced alkylamines in the urine. The way in which the alkylamines are formed from the formamides is unclear. It is unlikely that direct hydrolysis of the formamide molecule occurs, as NMF and DMF are both resistant to hydrolysis catalyzed by liver or plasma enzymes (C. Brindley and D. Ross, unpublished observation). Indeed, the observation of a large primary H/D kinetic isotope effect on the metabolism of NMF to methylamine when $ODCNHCH_3$ and $OHCNHCD_3$ are coadministered [k(formyl$-H$)/k(formyl$-D$) = 5.5. ± 0.2] shows that NMF is not hydrolyzed to methylamine *in vivo* but rather that cleavage of the formyl C-H bond is involved (M. D. Threadgill, P. Kestell and A. Gescher, unpublished observation). It is therefore likely that the product of alkylformamide oxidation or, indeed, its conjugation product *S*-(N-alkylcarbamoyl)glutathione or its cysteinylglycine, cysteine or mercapturic acid derivatives are the immediate precursors of the alkylamines found in the urine.

Among the amides studied here, NMA is the only one incapable, in principle, of undergoing oxidation or dehydrogenation in its carbonyl moiety. It was metabolized predominantly to its hydroxymethyl amide. The major metabolic pathway of DMF in rodents is also N-methyl C-hydroxylation (Kestell *et al.*, 1986a; Scailteur and Lauwerys, 1984). This is remarkable in view of the fact that DMF possesses a formyl group which could be oxidized. However, oxidation at the formyl carbon does not appear to occur in DMF. On the basis of the demonstration of the very low toxicity *in vivo* of a related carbinolamide, N-(hydroxymethyl)formamide (Cooksey *et al.*, 1983), which has been found to be a minor urinary metabolite of NMF (Kestell *et al.*, 1985), we suggest that metabolic N-methyl C-hydroxylation (fig. 5B) is a detoxification pathway in this series. Therefore, the results of the study described here lead to the proposition that two metabolic pathways of N-alkylformamides can be distinguished (fig. 5): either hydroxylation at the α-carbon of the N-alkyl group, which probably constitutes a detoxification reaction, or metabolic oxidation of the formyl moiety, which is a prelude to the generation of the hepatotoxic lesion.

In antitumor tests in mice of a large number of amides, only NMF possessed highly significant activity in several murine tumors (Gate *et al.*, 1986). Both NEF and DMF were only marginally inhibitory of tumor growth, each in one mouse tumor model. F and NMA lacked completely antitumor activity. Because, as shown in this paper, NMF and NEF (amongst the compounds examined) both exhibit hepatotoxicity and are metabolized significantly to *S*-(N-alkylcarbamoyl)-mercapturic acid but only NMF is an effective antitumor agent in mice, it would appear that the biochemical mechanisms of hepatotoxicity and antitumor activity are different.

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PUBLICATION 23

The Metabolism of *N*-Methylformamide in Mice; Primary Kinetic Deuterium Isotope Effect and Identification of *S*-(*N*-Methylcarbamoyl)glutathione as a Metabolite

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Metabolism of N-Methylformamide in Mice: Primary Kinetic Deuterium Isotope Effect and Identification of S-(N-Methylcarbamoyl)Glutathione as a Metabolite[1]

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ABSTRACT

S-(N-Methylcarbamoyl)glutathione has been identified by cesium ion liquid secondary ion mass spectrometry as a biliary metabolite in mice of the experimental antitumor agent and hepatotoxin N-methylformamide. Metabolism of N-methylformamide to urinary methylamine, urinary N-acetyl-S-(N-methylcarbamoyl)-cysteine and biliary S-(N-methylcarbamoyl)glutathione was found to be subject to large intermolecular primary kinetic isotope effects when hydrogen was replaced by deuterium in the formyl group (k_H/k_D = 5.5 ± 0.2, 4.5 ± 1.0 and 7 ± 2, respectively), as shown by mass spectrometry of derivatives of these metabolites. These values indicate the existence of a common metabolic precursor for each of these metabolites. In particular, methylamine is shown not to arise from simple enzymatic hydrolysis of N-methylformamide but is associated with an oxidative process. Therefore, it is highly likely that N-methylformamide is oxidized and conjugated to form S-(N-methylcarbamoyl)glutathione which is metabolized further to N-acetyl-S-(N-methylcarbamoyl) cysteine. Either of these thiocarbamates could be hydrolyzed to give the parent thiol and the observed metabolic end products, methylamine and carbon dioxide. The presence of deuterium in the formyl moiety of N-methylformamide reduced markedly the hepatotoxicity of the compound, as shown by measurements of the activities of appropriate hepatic enzymes in plasma.

NMF ($OHCNHCH_3$; NSC 3051; fig. 1) is a powerful solvent with antineoplastic activity against implanted rodent tumors (Clarke *et al.*, 1953; Gescher *et al.*, 1982). Of a large series of analogs tested in these murine systems, only NMF showed consistent activity (Gate *et al.*, 1986). NMF has been the subject of clinical evaluation (Laird Myers *et al.*, 1956; McVie *et al.*, 1984; Eisenhauer *et al.*, 1986) and has shown hepatotoxicity both in humans (Laird Myers *et al.*, 1956) and in rodents (Whitby *et al.*, 1984; Langdon *et al.*, 1985). It is likely that this toxicity is mediated *via* one or more metabolites of NMF (Pearson *et al.*, 1987; Whitby *et al.*, 1984). It has also been suggested that the hepatotoxicity of the important industrial solvent (DMF) may be associated with its metabolism to NMF (Kimmerle and Eben, 1975a,b; Lundberg *et al.*, 1981), although a recent study has shown that the latter secondary amide is at most a very minor metabolic product when high doses of DMF are administered to mice (Kestell *et al.*, 1986a). NMF is metabolized extensively in mammals and the following urinary metabolites have been identified: N-(hydroxymethyl)formamide (mouse) (Kestell *et al.*, 1985b), methylamine (mouse and rat) (Kestell *et al.*, 1985a,b) and N-acetyl-S-(N-methylcarbamoyl)cysteine (mouse, rat and humans) (Kestell *et al.*, 1986b; Tulip *et al.*, 1986). Additionally, $^{14}CO_2$ was found in the breath of mice which had received [^{14}C]NMF (Kestell *et al.*, 1985b) and some 26% of the parent amide is excreted unchanged in this species (Brindley *et al.*, 1982).

In a limited structure-toxicity study, we have reported recently (Kestell *et al.*, 1987) the observation of a qualitative relationship between the hepatotoxic potential of formamides and the presence of an S-(N-alkylcarbamoyl)mercapturic acid as a urinary metabolite. Therefore, the metabolic pathway of

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ABBREVIATIONS: NMF, N-methylformamide; DMF, N,N-dimethylformamide; EI, electron impact; BPt, boiling point; HPLC, high-pressure liquid chromatography; LSIMS, liquid secondary ion mass spectrometry; TLC, thin-layer chromatography; CI, chemical ionization; SDH, sorbitol dehydrogenase; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

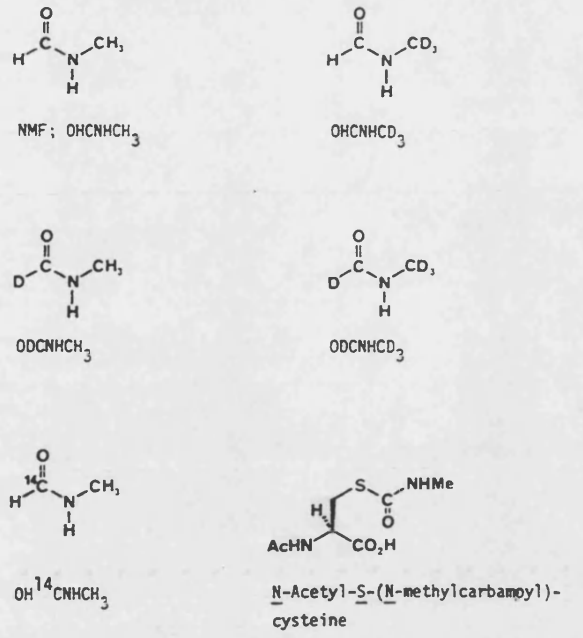

Fig. 1. Structures of synthetic compounds and isotopomers used in this study.

NMF which leads to the mercapturic acid appears to be the prime candidate for the route which generates the hepatotoxic intermediate. The chemical identity of this material and other details of the sequence of metabolic events leading from NMF to the mercapturic acid are unknown and difficult to establish directly as the reactive intermediate is likely to be a very short-lived species. Experiments are reported here which are designed to elucidate these details with the use of deuterated analogs of NMF. In particular, the following three hypotheses have been tested: 1) *S*-(N-methylcarbamoyl)glutathione is a precursor of the urinary mercapturic acid and is itself excreted in the bile; 2) the metabolic cleavage of the C-H bond in the formyl group of NMF is the rate-determining step in the metabolism of NMF to the thiocarbamates and to methylamine; and 3) the metabolic removal of the formyl hydrogen is involved in the genesis of the hepatotoxic lesions caused by NMF. Overall, it was hoped that this work may contribute to an understanding of the mechanisms of toxicity and of antitumor activity of NMF and perhaps to the design of a therapeutic analog in which these activities are separated.

Methods

Materials. The radiochemical starting material ($H^{14}CO_2^-$, Na^+) was purchased from Amersham International P.L.C. (Amersham, U.K.). Deuterated starting materials and other chemicals were purchased from Aldrich Chemical Company Ltd. (Gillingham, Dorset, U.K.) or from Aldrich Chemical Company (Milwaukee, WI). N-[methyl-^{14}C]formamide ($OH^{14}CNHCH_3$) and N-(trideuteromethyl)formamide ($OCHNHCD_3$) were prepared as described previously (Threadgill and Gate, 1983) and the latter was found by EI mass spectrometry to be > 98% labeled in the methyl group. N-(trideuteromethyl)-deuteroformamide ($ODCNHCD_3$) was prepared as follows: sodium (161 mg, 7 mmol) was dissolved in anhydrous methanol (15 ml; freshly distilled from magnesium methoxide) and the solution of sodium methoxide was cooled to 0°C. Trideuteromethylamine hydrochloride ($CD_3NH_3^+$ Cl^-; 492 mg, 7 mmol) was added, followed after 20 min by methyl deuteroformate (1 ml). The reaction mixture was stirred for 16 hr before being filtered. Distillation (Kugelrohr) gave the product as a colorless liquid (70 mg, 22%) with BPt_{760} 183–187°C [literature BPt_{760} 180–185°C for protio compound (Weast, 1977)]. This material was found by EI mass spectrometry to consist of a mixture of 55% $C_2H_2D_3NO$ and 45% C_2HD_4NO. High field proton NMR analysis of this material indicated that the trideutero species corresponded to $OHCNHCD_3$ whereas the tetradeutero molecules were labeled at both the methyl and the formyl positions. N-Methyldeuteroformamide ($ODCNHCH_3$) was synthesized thus: methyl deuteroformate ($ODCOCH_3$) was added to anhydrous methanol (20 ml) which had been saturated with gaseous methylamine (CH_3NH_2) at 0°C. This mixture was stirred for 16 hr. Distillation (Kugelrohr) furnished N-methyldeuteroformamide (1.02 g, 75%) as a colorless liquid BPt_{770} 191–194°. EI mass spectrometry showed the material to be > 98% $ODCNHCH_3$. Infrared spectrum (liquid film) 3300, 2950, 2150 and 1650 cm^{-1}.

HPLC. HPLC purification of the N-ethoxycarbonylglutathione conjugate of NMF isolated from bile was performed using a Beckman model 342 instrument, equipped with a reverse phase 5 μM Ultrasphere ODS column (15 cm × 4.6 mm internal diameter, Rainin Instruments, Berkeley, CA). The mobile phase consisted of a linear 20 min gradient from 20 to 70% methanol in water, with 1% acetic acid throughout. The mobile phase was maintained at 70% methanol for a further 10 min. The flow rate was held at 1.0 ml min^{-1} and 1-min fractions were collected. Under these conditions the ethoxycarbonyl-, ethoxycarbonyl methyl ester- and ethoxycarbonyl dimethyl ester derivatives of the NMF conjugate eluted in fractions 5 to 6, 8 to 9 and 10 to 11, respectively. The recovery of radioactivity in the HPLC eluate was > 95% of that applied to the column. Detection was carried out by liquid scintillation counting using a Beckman LS-7500 counter. HPLC equipment and conditions for the analysis of the urinary metabolites were as described previously by us (Kestell *et al.* 1986b) except that the mobile phase for the purification of N-methyl-2,4-dinitro-aniline was methanol-water (2:1 v/v) and UV absorption detection was carried out at 346 nm.

Mass spectrometry. LSIMS of the derivatized glutathione conjugates was performed on a Kratos MS-50S mass spectrometer, equipped with a 23-kG magnet and a postacceleration detector which was operated at −10 kV. Samples were dissolved in a glycerol matrix containing HCl and NaCl (to increase the intensity of $[M + H]^+$ and $[M + Na]^+$ species) and ionization was achieved by bombardment with a 1.0-μA primary beam of Cs^+ ions (Falick *et al.*, 1986). Chemical ionization mass spectrometry was carried out on a VG70-70 instrument as described previously (Kestell *et al.*, 1986b). EI mass spectrometry of deuterated analogs of N-methyl-2-4-dinitroaniline was carried out using a VG Micromass 12B instrument operating at an electron energy of 70 eV with a probe temperature of 250°C. The exact isotopic composition of the mixtures of the deuterated analogs which were administered to mice was determined by EI mass spectrometry using either a VG Micromass 12B or a VG 70-70H instrument.

Animals and treatment procedures. NMF or its deuterated or ^{14}C-analogs (6.8 mmol kg^{-1} in 200 μl of saline) were administered to male BALB/c mice (20–25 g) i.p. The total dose corresponds to the optimum antitumor dose (Gate *et al.*, 1986). Animals were sacrificed by cervical dislocation.

Identification of the biliary glutathione conjugate and determination of the primary kinetic isotope effect on its formation. Mice received NMF or its deuterated analogs together with $OH^{14}CNHCH_3$ as tracer (final specific activity, 30 mCi $mmol^{-1}$). In experiments designed to elucidate the position of the link of the NMF-derived moiety to glutathione, separate groups of four mice received either $OHCNHCH_3$ or $ODCNHCH_3$. In order to determine the magnitude of the primary kinetic isotope effect on the formation of this conjugate, a further group of mice received a mixture comprising $OHCNHCH_3$ (59%), $OHCNHCD_3$ (22.8%) (a byproduct of the synthesis of $ODCNHCD_3$) and $ODCNHCD_3$ (18.2%). Mice were sacrificed 4 hr after administration of NMF and bile was then collected *via* puncture of the gall bladder with a syringe.

Aliquots of bile (50 μl) were treated with a solution of ethyl chloro-

formate (100 μl) in 0.1 M phosphate buffer (pH 9.0, 3.0 ml). Excess ethyl chloroformate was removed by extraction with dichloromethane (5 ml) and the residual aqueous portion was acidified (to pH < 2) and applied to a prewashed C_{18} Sep-Pak cartridge (Waters Associates, Milford, MA). The cartridge was rinsed with water and the ethoxycarbonyl derivatives were eluted with methanol and evaporated to dryness under a stream of nitrogen. The residues were subjected to analysis by HPLC. Fractions 5 to 7 (which contained the majority of the radioactivity) were pooled and processed, as described above, on a second Sep-Pak cartridge. The purified conjugates thus obtained were then treated with anhydrous methanolic hydrogen chloride at ambient temperature for 2 hr. The resulting methyl esters were purified further by HPLC, as described above, and fraction 10 was collected for mass spectrometric analysis. The precise isotopic composition of the fully derivatized NMF-glutathione conjugates, isolated from each of the separate studies, was determined under LSIMS conditions by sweeping the accelerating potential of the spectrometer repetitively to bring into focus the mass range 485 to 495. The duration of each limited mass scan was 10 sec and measurements of deuterium content were based on the $[M + Na]^+$ cluster (at m/z 487 in the spectrum of the unlabeled conjugate) using the mean of 10 scans across this peak.

Study on the metabolism of NMF to N-acetyl-*S*-(N-methylcarbamoyl)cysteine. Collections of urine (24 hr) were made from three mice which had been given a mixture of $OHCNHCD_3$ (50%) and $ODCNHCH_3$ (50%). Each sample of urine was freeze-dried and the residues were subjected to preparative TLC as described previously (Kestell *et al.*, 1986b). The isolated mercapturic acid from each sample was esterified using methanolic hydrogen chloride (Kestell *et al.*, 1986b) and the resulting mixtures of isotopomers of N-acetyl-*S*-(N-methylcarbamoyl)cysteine methyl ester were subjected to CI mass spectrometry in order to determine the isotopic composition of the N-methyl groups. Averages of five spectra were taken for each sample.

Study on the metabolism of NMF to methylamine. Urine was collected from each of three male mice for 24-hr periods both before and after administration of a mixture of $OHCNHCD_3$ (47%) and $ODCNHCH_3$ (53%). To each urine sample (*ca.* 1 ml) was added 2 M ethanolic 2,4-dinitrofluorobenzene (2 ml) and the mixtures were allowed to stand for 1 hr at ambient temperature before addition of saturated aqueous sodium chloride (1 ml). Each sample was then extracted with diethyl ether (2 × 5 ml). The organic extracts were washed with 2.5 M aqueous sodium hydroxide (2 × 2 ml) and with saturated aqueous sodium chloride (2 ml) before being dried with anhydrous sodium sulfate and filtered. The solvent was then evaporated from each filtrate under reduced pressure. An aliquot of the residue from each sample was then assayed for total N-methyl-2,4-dinitroaniline by HPLC to determine the contribution of endogenous CH_3NH_2 to the total methylamine in the urine samples from animals after administration of NMF. The N-methyl-2,4-dinitroaniline from the urine of each treated animal was purified by preparative TLC [2 mm thick silica gel 60 plates, Merck, Darmstadt, FRG, dichloromethane-methanol, 9:1 (v/v)], followed by HPLC. Evaporation of the eluent from appropriate fractions gave the pure mixtures of isotopic analogs as yellow solids, the compositions of which were then determined by EI mass spectrometry.

Studies on the effects of deuteration on hepatotoxicity of NMF. Single doses of $OHCNHCH_3$ and $OHCNHCD_3$ (100 and 200 mg kg^{-1}) and of $ODCNHCH_3$ (100, 200 and 300 mg kg^{-1}) were administered i.v. in saline (200 μl) and control mice received saline (200 μl) alone. Twenty four hours after administration of the dose, the animals were anesthetized with diethyl ether and exsanguinated by cardiac puncture into syringes containing aqueous sodium heparin (2500 U ml^{-1}; 50 μl). Plasma was obtained from each sample by centrifugation. The activity of SDH was determined by the method of Rose and Henderson (1975) and those of AST and ALT were determined by the method of Kachmar and Moss (1976). Statistical comparison was effected using the Mann-Whitney *U* test.

Results

The bile obtained from mice which had received $OH^{14}CNHCH_3$ contained a radioactive metabolite which was derivatized by treatment with ethyl chloroformate and the products were analyzed by HPLC. The exemplary chromatogram (fig. 2A) shows that a single radioactive component was present (fraction 5). This material was collected, esterified and analyzed in the same HPLC system (fig. 2B). One major, less polar, radioactive product (fraction 10) was observed, together with two minor components arising from incomplete esterification of the sample. The major product was isolated and subjected to structural analysis by Cs^+ ion LSIMS. The resulting mass spectrum (fig. 3) contained three prominent ions which correspond to the $[M + H]^+$ (m/z 465), $[M + Na]^+$ (m/z 487) and $[M + K]^+$ (m/z 503) species of a compound of MW 464. This molecular weight corresponds to the sum of those of NMF (59) and N-(ethoxycarbonyl)glutathione dimethyl ester (407) minus two hydrogens.

In order to determine to which position in the NMF moiety the glutathione was attached, the conjugate excreted in the bile of mice which had received $ODCNHCH_3$ was derivatized, purified and analyzed, similarly. The partial mass spectra reproduced in figure 4 are representative of those arising from the fully derivatized glutathione conjugates from $OHCNHCH_3$ and $ODCNHCH_3$ (fig. 4, A and B, respectively). The strong simi-

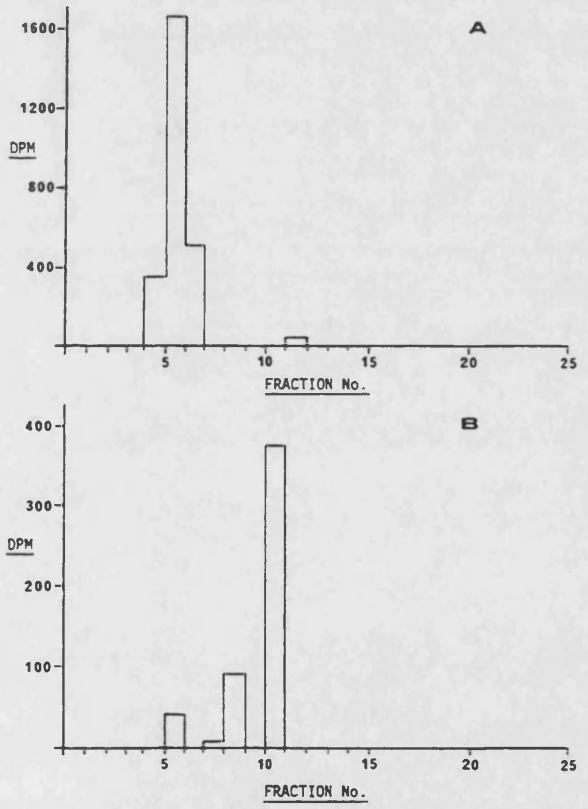

Fig. 2. A, reverse-phase high-pressure liquid chromatogram of bile of mice given $OH^{14}CNHCH_3$. After treatment of a sample of bile with ethyl chloroformate, metabolites were extracted and analyzed by HPLC as described under "Methods." Fractions (1 ml) were collected for radioactivity measurements. B, high-pressure liquid chromatograms of pooled fractions 4–6 from A, after treatment with methanolic hydrogen chloride.

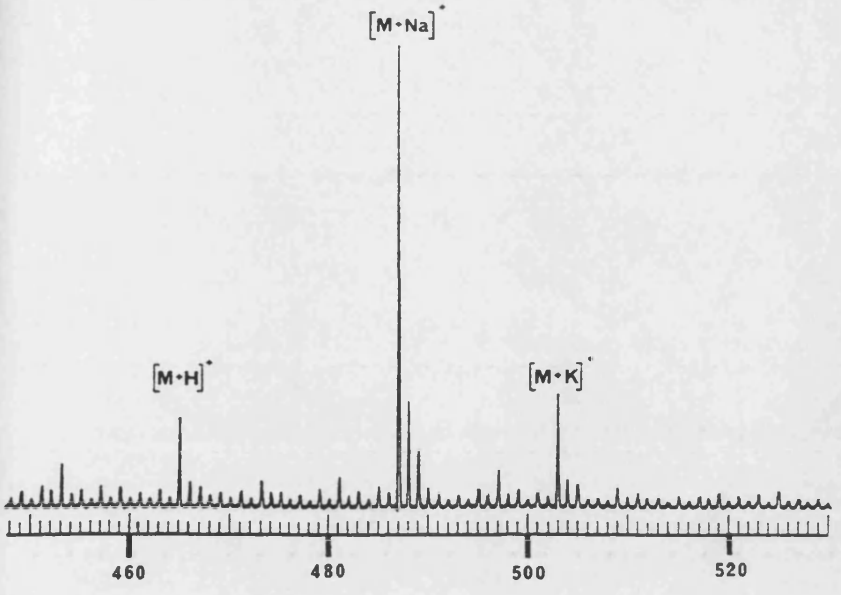

Fig. 3. Molecular ion region of the LSIMS spectrum of the fully derivatized NMF-glutathione conjugate as isolated by HPLC (fraction 10, fig. 2B).

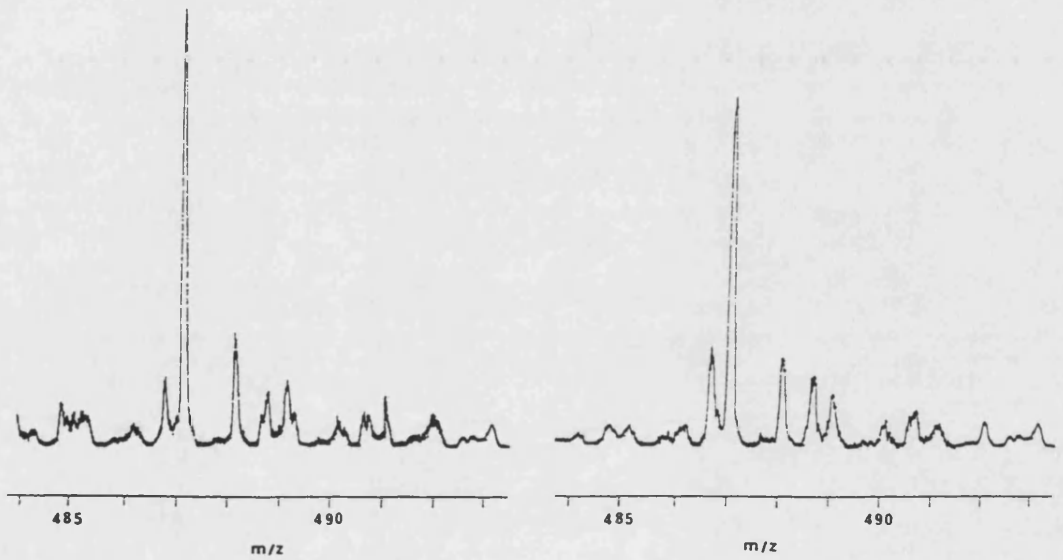

Fig. 4. A, the $[M + Na]^+$ region of the LSIMS spectra of the fully derivatized NMF-glutathione conjugates isolated from the bile of mice which had received $OHCNHCH_3$ (A) or $ODCNHCH_3$ (B).

larity in relative intensities of the $[M + Na]^+$ ions at m/z 487 and m/z 488 in the spectra of the two conjugates is indicative of the total loss of deuterium from the $ODCNHCH_3$ during the metabolic process to give the glutathione conjugate. Thus, the NMF moiety must be linked exclusively through the formyl group to glutathione. The structure of the fully derivatized conjugate, as determined in this study, is shown in figure 5.

The magnitude of the apparent primary kinetic deuterium isotope effect associated with formation of *S*-(N-methylcarbamoyl)glutathione from NMF labeled with deuterium at the formyl position was estimated by administering a known mixture of $OHCNHCH_3$, $OCHNHCD_3$ and $ODCNHCD_3$ to mice. Bile was collected from the animals, as before, and the conjugate was converted to its ethoxycarbonyl dimethyl ester derivative. Based on the relative intensities of the ions at m/z 487 and m/z 490 in the LSIMS spectrum of this derivatized conjugate, corresponding to $[M + Na]^+$ ions for the $—SCONHNCH_3$ and $—SCONHCD_3$ species, respectively (fig. 6), and taking into account the $GSCONHCD_3$ generated from the impurity

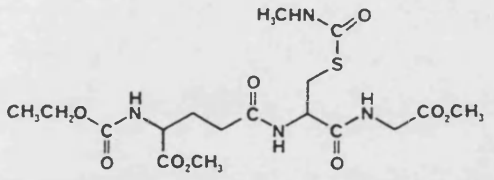

Fig. 5. Structure of the N-(ethoxycarbonyl) dimethyl ester derivative of the glutathione conjugate derived from NMF.

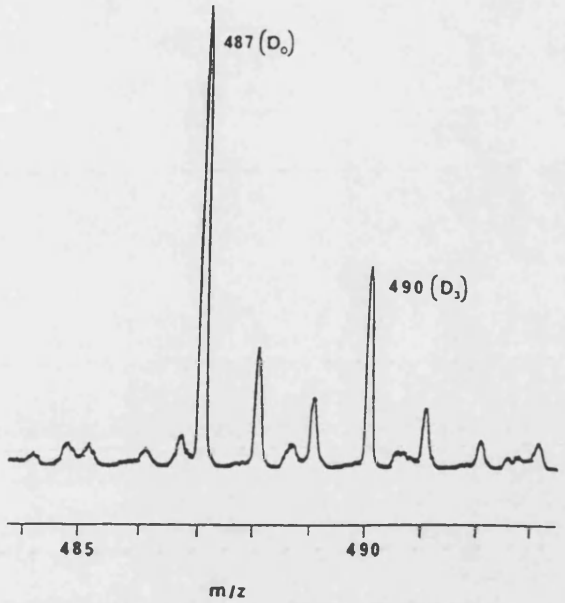

Fig. 6. The $[M + Na]^+$ region of the LSIMS spectrum of fully derivatized *S*-(N-methylcarbamoyl)glutathione isolated from the bile of mice which had received a mixture of $OHCNHCH_3$ and $ODCNHCD_3$.

$OHCNHCD_3$, it could be calculated that the apparent kinetic isotope effect associated with this metabolic process *in vivo* (k_H/k_D) is 7 ± 2 (n = 3) as referred to the formyl carbon-hydrogen bond. In this experiment, the presence of a $—SCONHCH_3$ group in the conjugate reports on the metabolism of $OHCNHCH_3$ and the $—SCONHCD_3$ reports on the metabolism of the $ODCNHCD_3$ and $OHCNHCD_3$.

The primary kinetic deuterium isotope effect on the metabolism of NMF to N-acetyl-*S*-(N-methylcarbamoyl)cysteine was evaluated by administration of a known mixture of $ODCNHCH_3$ and $OHCNHCD_3$ to mice. The mixtures of deuterated analogs of the mercapturic acid were isolated from urine from each mouse and were esterified by treatment with methanolic hydrogen chloride before CI mass spectrometric analysis (fig. 7). Comparison of the intensities of the ions at m/z 235 and m/z 238, corresponding to the molecular ions of N-acetyl-*S*-(N-methylcarbamoyl)cysteine methyl ester and N-acetyl-*S*-[N-(trideuteromethyl)carbamoyl]cysteine methyl ester, respectively, indicated the magnitude of the apparent primary kinetic isotope effect (k_H/k_D) for this metabolic process to be 4.5 ± 1.0 (n = 3). In this experiment, the mercapturic acid bearing the $SCONHCD_3$ reports on the metabolism of $OHCNHCD_3$ and that bearing the $SCONHCH_3$ group reports on the metabolism of $ODCNHCH_3$.

To evaluate the corresponding apparent primary kinetic isotope effect for the metabolism of NMF to methylamine, a similar mixture to that used for the N-acetylcysteine conjugate experiment was administered. Twenty four-hour urine collections were made both before and after the dose. Derivatization of the urinary methylamine was effected by treatment of the urine samples with ethanolic 2,4-dinitrofluorobenzene under neutral conditions. HPLC assay showed that endogenous methylamine (CH_3NH_2) in the control samples comprised < 5% of the total methylamine (CH_3NH_2 + CD_3NH_2) found in the urine after treatment of the animals; the contribution of endogenous methylamine to the total was therefore regarded as negligible. The N-methyl-2,4-dinitroaniline from the samples of urine after administration of NMF was purified by extraction into diethyl ether, followed by preparative TLC and HPLC. Based on the intensities of the ions in the EI mass spectra at m/z 197 and m/z 200, being the molecular ions for N-methyl-2,4-dinitroaniline and N-(trideuteromethyl)-2,4-dinitroaniline, respectively, an apparent kinetic isotope effect (k_H/k_D) of 5.5 ± 0.2 (n = 3) was observed for the metabolism of NMF to methylamine, depending on the presence of a C—H or a C—D bond in the formyl group of the substrate. Again, the $—NCD_3$ group reports on the metabolism of $OHCNHCD_3$ and the $—NCH_3$ group reports the production of methylamine from $ODCNHCH_3$.

The elevations of the activities of the hepatocellular enzymes SDH, AST and ALT in the plasma of mice which had received NMF or its isotopic analogs (fig. 8) indicate that the threshold dose of $OHCNHCH_3$ and $OHCNHCD_3$ for hepatotoxicity lies between 100 and 200 mg kg^{-1}, whereas that of $ODCNHCH_3$ is markedly higher and falls between 200 and 300 mg kg^{-1}.

Discussion

There is little doubt that the hepatotoxicity of NMF is associated with its metabolism (Whitby *et al.*, 1984; Pearson *et al.*, 1987). However, the structure of the hepatotoxic metabolite has hitherto eluded characterization. We have reported previously that NMF is metabolized (*inter alia*) to methylamine and N-acetyl-*S*-(N-methylcarbamoyl)cysteine (Kestell *et al.*, 1985b, 1986b). In this paper, the excretion of *S*-(N-methylcarbamoyl)glutathione as a biliary metabolite of NMF is demonstrated unambiguously. That the "NMF" moiety is linked through the formyl carbon to glutathione was shown by the complete loss of deuterium from $ODCNHCH_3$ during metabolic conjugation. Thus, the *S*-substitution is the same as that in the mercapturic acid derived from NMF and consequently it would appear that the glutathione conjugate characterized here is a metabolic precursor of the latter urinary conjugate *in vivo* (fig. 9).

In order to assess the importance of the metabolic cleavage of the formyl carbon-hydrogen bond on the metabolism and toxicity of NMF, the effect of replacement of C—H by C—D was studied. Carbon-deuterium bonds have a higher activation energy for cleavage than do carbon-hydrogen bonds, owing to the lower zero-point energy of the former (Alder *et al.*, 1971). If a carbon-hydrogen bond is cleaved during the rate-determining step of a multistep process, then substitution of deuterium for hydrogen will have the effect of slowing the rate of formation of all products (metabolites) downstream of this step. The formation of the glutathione conjugate of NMF was found to be subject to such an apparent intermolecular primary kinetic deuterium isotope effect in that it was slowed by a factor of 7 ± 2 when hydrogen in the formyl group of NMF was replaced by deuterium. A similarly significant kinetic isotope effect was observed for the formation of the mercapturic acid. These results confirm that both the *S*-substituted glutathione and the mercapturic acid lie downstream of the rate-limiting step in this metabolic pathway. Also implied is the conclusion that the formyl C—H bond is broken in this step. Interestingly, the formation of urinary methylamine is subject to an apparent kinetic isotope effect of the same magnitude when formyl-H is replaced by formyl-D in the substrate NMF. Hence, this amine is not the product of any biological hydrolysis of NMF as such

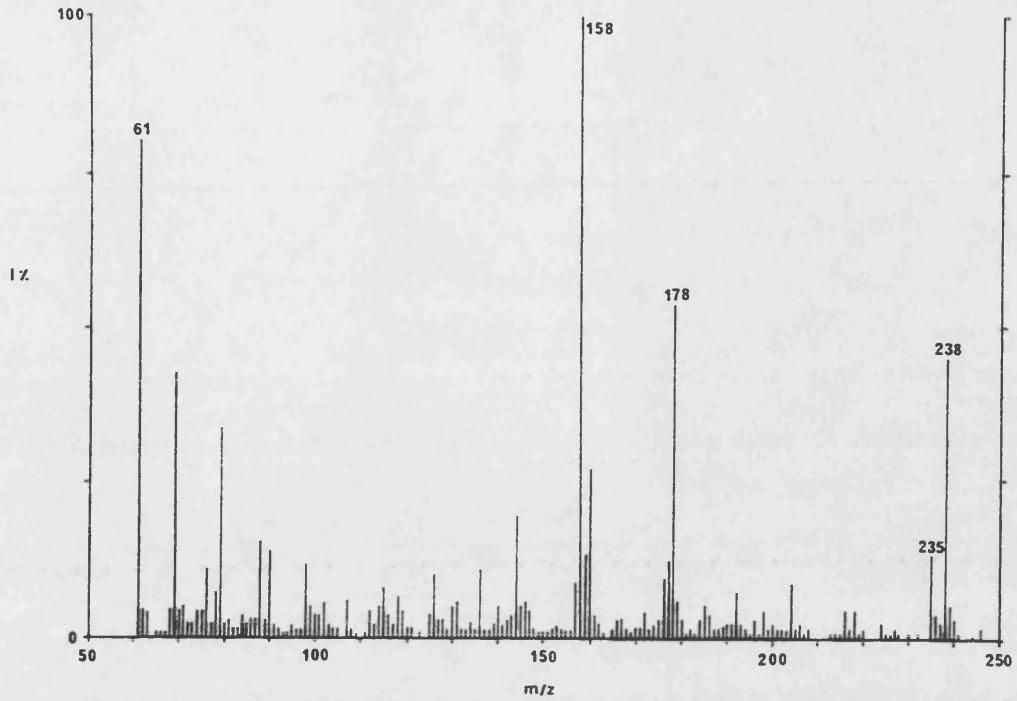

Fig. 7. Example of a CI mass spectrum of the methyl esters of N-acetyl-*S*-(N-methylcarbamoyl)cysteine isolated from the urine of mice which had received a 50:50 mixture of $ODCNHCH_3$ and $OHCNHCD_3$.

reactions necessarily would not involve cleavage of this formyl C—H bond and thus an hepatic formamidase similar to that reported by Shinohara and Ishiguro (1979) or to the bacterial N,N-dimethylformamidase described by Schär *et al.* (1986) is not involved in the metabolism of NMF. Therefore we propose that, in the metabolic sequence, NMF is first oxidized at the formyl group to give a short-lived reactive intermediate in which the formyl-H bond has already been broken (represented as ? in fig. 9) and that this compound is conjugated with glutathione either chemically or catalytically by a glutathione *S*-transferase. It is, of course, possible that the oxidation and conjugation steps are concerted, *i.e.*, that glutathione is a co-factor of the oxidizing enzyme and that there is no discrete intermediate (?). This glutathione conjugate would then be converted to the mercapturic acid in the usual way, presumably through the intermediacy of the cysteinylglycine and cysteine conjugates which are, as yet, undetected. The facile base-catalyzed chemical hydrolysis *in vivo* of any or all of these metabolites, including the postulated initial reactive intermediate, may well be responsible for the generation of the methylamine, as shown in figure 9. The $^{14}CO_2$ which is a metabolite of $OH^{14}CNHCH_3$ (Kestell *et al.*, 1985b) would be, in part, the by-product of this hydrolysis.

The observation and mechanistic significance of primary kinetic deuterium isotope effects occurring in biological systems has been recently reviewed briefly by Van Langenhove (1986) and, more extensively, by Pohl and Gillette (1985) and by Foster (1985). It has been shown that the intermolecular kinetic isotope effects on the oxidative demethylation catalyzed by cytochrome P-450, in rodent microsomes, of several substrates is low, with k_H/k_D frequently equal to unity (Miwa *et al.*, 1980). The corresponding intramolecular kinetic isotope effects (representing more closely the intrinsic effect) have been reported to lie in the range k_H/k_D = 1.45 to 3.9 (Miwa *et al.*, 1980; Hjelmeland *et al.*, 1977; Heimbrook *et al.*, 1984) and the intramolecular effect using a model porphyrin system is similar (Nee and Bruice, 1982). Whether cytochrome P-450 enzymes can catalyze the oxidation of N-formyl groups remains to be established. The mechanism of yeast formate dehydrogenase has been shown to involve hydride transfer from formate ion to NAD^+ in a process which is subject to a (necessarily intermolecular) kinetic deuterium isotope effect (k_H/k_D = 2.3) on replacement of substrate HCO_2^- by DCO_2^- (Blanchard and Cleland, 1980; Hermes *et al.*, 1984). 10-Methylacridinium ion has been claimed to be a useful model for this enzymic reaction in that it also oxidizes formate to carbon dioxide through a hydride transfer mechanism with a similar retardation when the substrate is deuterated at carbon (k_h/k_D = 2.7) (Hutchins *et al.*, 1986). However, NMF was not oxidized by this chemical hydride acceptor (M. D. Threadgill, unpublished observations), despite the apparent structural similarity of the formamide to formate. In view of the large intermolecular deuterium kinetic isotope effects demonstrated for the metabolism of NMF, it is unlikely that cytochrome P-450-containing enzymes catalyze the oxidation of the N-formyl group, although an isoenzyme not examined in previous kinetic isotope effect studies may be involved. Conversely, it may be that NMF is metabolized by another enzyme system related to formate dehydrogenase, such as monoamine oxidase or xanthine oxidase. The results of studies *in vitro* to determine the enzyme responsible for this oxidation will be presented later.

Primary kinetic isotope effects on toxicity also have been described. Deuterochloroform ($CDCl_3$) was reported to be some 50 to 70% less cytotoxic in rodents than protiochloroform ($CHCl_3$) (Branchflower *et al.*, 1984), indicating that metabolic cleavage of the carbon-hydrogen bond is involved in the for-

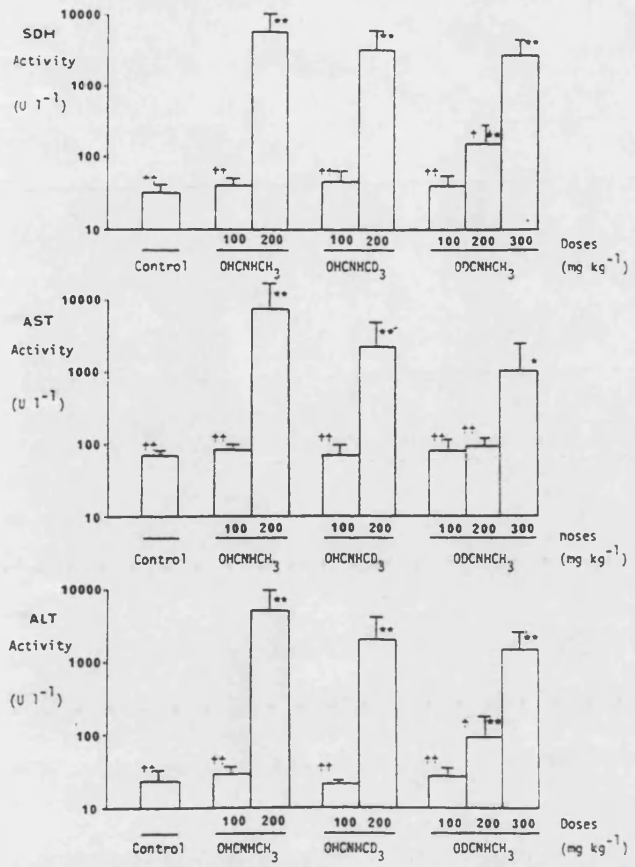

Fig. 8. Activities of SDH, AST and ALT in the plasma of mice 24 hr after doses of $OHCNHCH_3$, $OHCNHCD_3$ or $ODCHNCH_3$. Values are the mean ± SD. Significantly different from control at $P < .005$ (*) and $P < .002$ (**) (Mann-Whitney *U* Test). Significantly different from results from mice receiving 200 mg kg^{-1} $OHCNHCH_3$ at $P < .005$ (†) and $P < .002$ (††).

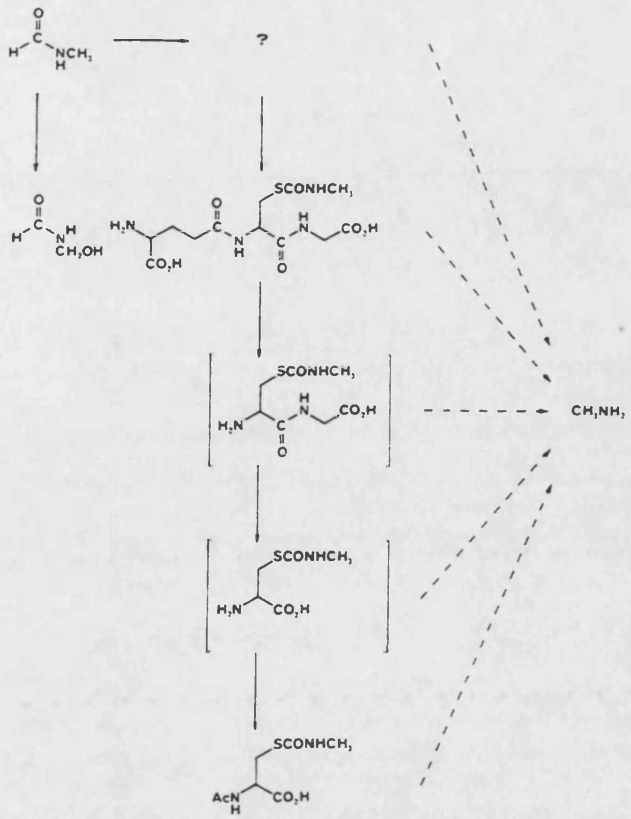

Fig. 9. Proposed pathway for the metabolism of NMF in mice.

mation of the ultimate toxic derivative. Conversely, tetradeutero-1,2-dibromoethane ($BrCD_2CD_2Br$) has been shown to be more genotoxic than the protio analog ($BrCH_2CH_2Br$) (White *et al.*, 1983), showing retardation of a competing detoxification pathway. In the present study, we have shown that the hepatotoxicity of NMF is reduced upon deuteration of the NMF in the formyl group. No such effect was in evidence for the trideuteromethyl analog. There appears to be a relationship between the hepatotoxicity of formamides and the metabolic generation of the corresponding N-acetyl-*S*-(N-alkylcarbamoyl)cysteine (Kestell *et al.*, 1987). These two pieces of evidence suggest strongly that either a *S*-(N-methylcarbamoyl)peptide or amino-acid metabolite or the (as yet) unknown reactive intermediate arising from oxidation of the formyl group of NMF (possibly methyl *iso*cyanate) may be the hepatotoxic entity. It is unlikely that methylamine is responsible for the damage to the liver, inasmuch as this amine is present in untreated animals, albeit in small amounts. Further pharmacological studies will show whether replacement of the formyl hydrogen by deuterium will lead to loss of antineoplastic potency similar to the diminution of hepatotoxicity caused by $ODCNHCH_3$.

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PUBLICATION 24

Activities of Serine Hydroxymethyltransferase in Murine Tissues and Tumours

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ACTIVITIES OF SERINE HYDROXYMETHYLTRANSFERASE IN MURINE TISSUES AND TUMOURS

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SUMMARY

The specific activity of the enzyme serine hydroxymethyltransferase (EC 2.1.2.1) was determined in various murine, rat and human tumour cell lines. The activities of the enzyme were also investigated in tissues of non-tumour bearing DBA/2 mice and BALB/c mice bearing the PC6 ascites tumour. The highest enzyme activity in the murine tissues was found in the liver and then the kidneys. The enzyme was present in all the tissues assayed. The activities of enzyme found in the tumours varied considerably, with the PC6 ascites, Walker 256 and Lewis lung cells, being the highest.

INTRODUCTION

The enzyme serine hydroxymethyltransferase (SHMT, EC 2.1.2.1) catalyses the interconversion of L-serine and glycine, in the presence of *dl*-L-tetrahydrofolic acid (FH_4), generating a one carbon unit in the form of N^5,N^{10}-methylenetetrahydrofolate (CH_2–FH_4). This one carbon unit may be used for the biosynthesis of purines, pyrimidines and, as N^5-methyltetrahydrofolate, methionine [7]. SHMT has been found in several tumour cells [9]. Recently Snell and Weber have investigated the enzymes of L-serine metabolism in rat hepatomas [10]. Unlike the other enzymes of L-serine catabolism, only SHMT was present in the hepatomas. They suggest that this imbalance of enzymes is due to L-serine being biosynthesised preferentially for formation of nucleosides in the tumour cells. This has been

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confirmed by Burkin and Draudin-Krylenko who found a relationship between the activities of SHMT in Friend mouse leukaemia and Pliss' rat lymphosarcoma with the rate of development of the tumour [1]. These results suggest that SHMT may be a good target for cancer chemotherapy.

In an attempt to determine whether the activities of enzyme found in various tumour cells are elevated when compared to normal cells and whether the enzyme activity can be related to the tumour type, the activity of SHMT in various malignant and normal tissues has been investigated.

MATERIALS AND METHODS

All reagents were of Analar grade and were purchased from BDH Chemicals (Atherstone, Warwickshire, U.K.) or Sigma (Poole, Dorset, U.K.). L-[3-^{14}C]Serine (53 mCi mmol^{-1}) was obtained from Amersham International (Amersham, Buckinghamshire, U.K.). Ascites tumours were removed from the peritoneal cavity of mice or rats, washed with 0.9% sodium chloride and resuspended in erythrocyte cell lysis buffer (ammonium chloride 7.5 gl^{-1} in 0.16 M Tris–HCl pH 7.2). The suspension was sedimented at 2000 × *g* for 10 min, washed twice with 0.9% sodium chloride, and the cells were disrupted as below. Internal organs and solid tumours were freshly removed, homogenised and treated as for ascitic fluid. Murine bone marrow was removed by the method of Oliver and Goldstein [5].

The cells were disrupted in a minimum volume of 100 mM Tris–HCl buffer at pH 7.1 at 10 kHz for 3 × 10 s using a MSE sonic oscillator. The suspension was sedimented at 3000 × *g* for 1 h and the pellet was discarded. The SHMT activity was assayed using the method of Taylor and Weissbach using L-[3-^{14}C]serine as the substrate for the enzyme and measuring the rate of formation of [^{14}C]formaldehyde. The number of counts per minute was recorded on a Beckman LS-230 scintillation counter. Protein was determined by the method of Lowry et al [4], using bovine serum albumin as standard.

The TLX5 lymphoma and PC6 plasmacytoma ascitic fluid were obtained from CBA and BALB/c mice respectively, supplied by Mr. D. Chubb, Aston University. The Lewis lung carcinoma solid tumour was obtained from C57BL/6 mice, the Walker 256 ascites and solid tumour were obtained from Wistar rats, and the sarcoma 180 solid tumour was obtained from CRH mice, all supplied by Dr. R. Fenton, Glaxo Group Research, Greenford, U.K. HT29 human colon carcinoma cells and Colon 26 cells were obtained from Dr. A. Baxter, Glaxo Group Research. The murine L1210 leukaemia cells derive from Flow Laboratories and the L1210 MTX, originally obtained from Flow Laboratories, were made resistant to methotrexate. O^6-Alkyl-(methyl)guanine-DNA-methyltransferase (O^6-MeGMT) proficient and deficient [2] Raji cell lines were also used in this study.

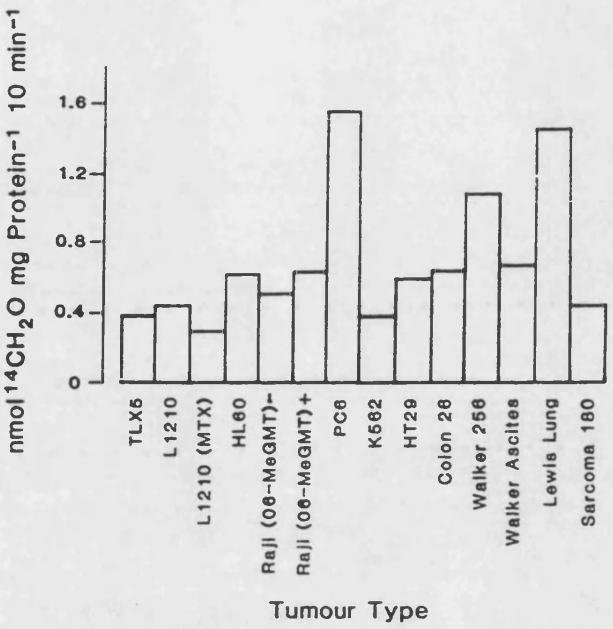

Fig. 1. The relative rate of formation of [^{14}C]formaldehyde by tumour cells.

RESULTS

The production of [^{14}C]formaldehyde from L-[3-^{14}C]serine proceeded linearly for 20 min, with enzyme concentrations up to 0.3 mg of protein per incubation. The SHMT activity for the tumour cells investigated was determined by measuring the rate of formation of [^{14}C]formaldehyde over a 20-min period. Enzyme activity of the tissues was measured by determining the quantity of [^{14}C]formaldehyde produced during a 10-min incubation. Duplicate determinations agreed within 5%.

The relative rates of formation of [^{14}C]formaldehyde by tumour cells is presented in the form of a histogram in Fig. 1 (the enzyme activity is the linear regressed value for nmole [^{14}C]formaldehyde per mg protein in 10 min). The levels of SHMT activity for the different tissues of non-tumour-bearing DBA/2 and of BALB/c (bearing PC6 ascites) mice are shown in the form of histograms in Fig. 2.

DISCUSSION

SHMT is present in all of the tumour cells assayed in this study. Similar specific activities were obtained for all the cell types investigated with the

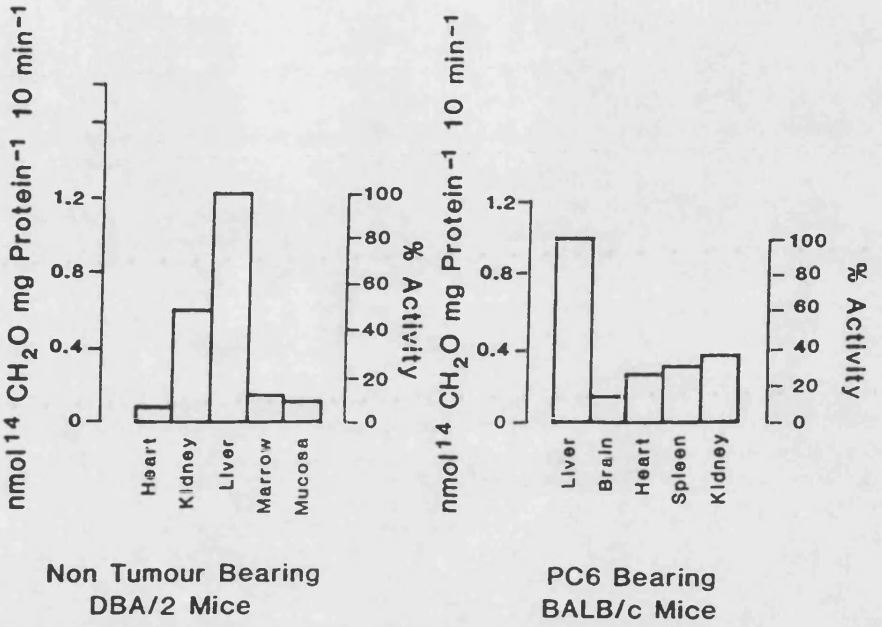

Fig. 2. The relative rate of formation of [^{14}C]formaldehyde by murine tissues.

exception of the PC6 ascites, Walker 256 and Lewis lung cells, which have elevated levels of activity. Little difference in activity was observed from the methotrexate-sensitive and -resistant L1210 cell lines. The presence or absence of O^6-MeGMT also does not appear to influence the activity of the enzyme.

The activity of SHMT found in the different organs of the BALB/c bearing PC6 ascites and the non-tumour-bearing DBA/2 mice are similar to those previously reported [8,13]. The highest activities of the enzyme are found in the liver, followed by the kidneys. SHMT was found to be present in all the tissues assayed. The levels of activity of SHMT found in the murine liver appears to be higher than the levels found in many of the tumour cells assayed, whereas the other murine tissues have levels comparable to the majority of the tumour cell lines. The high activity of SHMT in the kidneys is to be expected as the rat kidney has been shown to have significant levels of the enzyme in the proximal segment of the nephron [3], allowing the independent synthesis of L-serine from glycine [6].

No strong trend could be observed in the levels of activity of SHMT in the cell types surveyed. However, the presence of enzyme activity in all the tumour cell lines, together with the role of the enzyme in supplying C_1 units for biosynthesis of nucleosides during, for example, cell proliferation, suggests that SHMT may be an apposite target for anticancer therapy. The

results of a programme of synthesis and evaluation of compounds designed to inhibit SHMT will be reported elsewhere [12].

ACKNOWLEDGEMENTS

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Structural Studies on Bio-Active Compounds. Part 6. Determination of the Sites of Protonation on Three 2,4-Diaminopyrimidines of Pharmaceutical Importance by ^{13}C NMR and ^{1}H NMR

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Structural Studies on Bio-active Compounds. Part 6.[1] Determination of the Sites of Protonation on Three 2,4-Diaminopyrimidines of Pharmaceutical Importance by Proton-coupled ^{13}C and ^{1}H Nuclear Magnetic Resonance Spectroscopy

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The protonation of three 2,4-diaminopyrimidines of interest as antitumour agents has been studied by proton-coupled ^{13}C n.m.r. and by ^{1}H n.m.r. spectroscopy in solution in dimethyl sulphoxide. Assignment of ^{13}C resonances was achieved through chemical-shift considerations and through ^{13}C–^{1}H coupling patterns, supported in one case by a DEPT experiment and by heteronuclear decoupling by selective irradiation in the proton spectrum. In each case, first protonation occurs at pyrimidine N-1 with the second, much weaker, basic site of 2,4-diamino-5-(3-amino-4-chlorophenyl)-6-ethylpyrimidine being at the aniline amino group. The observations are consistent with current hypotheses of structural requirements for inhibitory activity against dihydrofolate reductase.

The 2,4-diaminopyrimidines, and particularly 2,4-diamino-5-(4-chlorophenyl)-6-ethylpyrimidine (pyrimethamine) (1), are important in the control of infectious disease through their potent inhibition of dihydrofolate reductase (DHFR).[2] Inhibition of the corresponding mammalian enzyme with the folate analogue methotrexate has also proved a successful strategy in the chemotherapy of neoplastic disease.[3] Recently, 2,4-diamino-5-(3-azido-4-chlorophenyl)-6-ethylpyrimidine (MZP) (2) has entered clinical trial as an antitumour agent as its monoethanesulphonate salt (MZPES). Protonation at the ring nitrogen N-1 at physiological pH (pH 7.4) has been suggested to be of considerable importance in the inhibitory binding of 2,4-diaminopyrimidines to the active site of DHFR,[4,5] although the site of protonation of the pyrimidines in solution has hitherto been inferred from solid-state crystallographic techniques.[6] It was therefore of interest to determine the sites of protonation of 2,4-diaminopyrimidines in solution through study of the ^{13}C n.m.r. spectra of these compounds in protonated and unprotonated forms.

The pyrimidines studied were two compounds of therapeutic importance (1) and (2) and 2,4-diamino-5-(3-amino-4-chlorophenyl)-6-ethylpyrimidine (3) which is a metabolite, photoproduct, and thermal degradation product[7] of compound (2). Chemical-shift data from ^{13}C n.m.r. experiments are potentially more useful than ^{1}H data for this study owing to the lack of protons bonded directly to pyrimidine ring carbons. Thus, ^{1}H n.m.r. spectroscopy can only report protonation of the pyrimidine ring at long range in these compounds. Confident assignment of the ^{13}C resonances is essential; hence proton-coupled ^{13}C spectra were obtained and, in one case, selective heteronuclear decoupling was employed. Dimethyl sulphoxide (DMSO) was used as spectroscopic solvent in most experiments, owing to the very poor solubility of the free bases of compounds (1)—(3) in water.

The proton-coupled ^{13}C n.m.r. spectral data of (1), the simplest of the three pyrimidines studied, is listed in Table 1 (numbering scheme shown in Figure 1). Assignment of the resonances corresponding to C-1″ and C-2″, the carbon atoms of the ethyl group attached to C-6 of the pyrimidine, was carried out in a straightforward manner from chemical-shift considerations with C-1″ resonating at δ 27.32 and C-2″ at δ 13.76. Confirmation was achieved by consideration of carbon–proton coupling. The signal from C-1″ is found to be a triplet of quartets with a one bond C–H coupling constant (^{1}J 126.7 Hz) typical for an sp^3-hybridised carbon atom; the two-bond coupling constant corresponding to coupling of the methyl protons with C-1″ (^{2}J 4.5 Hz) is also consistent with reported values for related systems.[8] The ^{1}J and ^{2}J values for the quartet of triplets from the methyl carbon (C-2″) are very similar to those of C-1″.

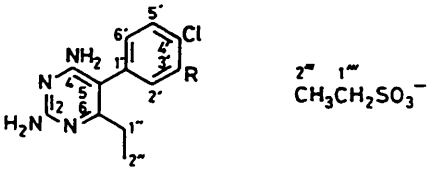


Figure 1. Numbering scheme for carbon atoms in pyrimidines (1: R = H), (2: R = N_3), and (3; R = NH_2) and in ethanesulphonate anion

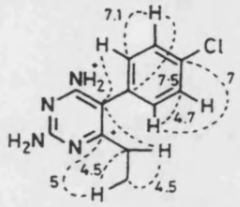
Assignment of the ^{13}C resonances from the benzene ring of (1) was carried out in the opposite order, *i.e.* consideration of multiplicity allowed the four resonances between δ 110 and δ 145 (the usual aromatic range) to be separated into groups of tertiary carbons (with coupling > 150 Hz) and quaternary carbon (all C–H coupling < 10 Hz). Careful comparison of chemical shifts with accepted typical values[8] then enabled individual assignment of ^{13}C resonances as in Table 1. The one-bond C–H coupling constants (^{1}J 161.0 and 165.4 Hz) correspond with the usual values for sp^2-hybridised carbon atoms. Longer range couplings (^{2}J 4.7 Hz and ^{3}J 7.5, 7 Hz) are also of typical magnitude for an aromatic ring, although $^{2}J_{C\text{-}2',3'\text{-}H}$ 7.1 Hz is unusually large[8] (Figure 2). Little has been published on the ^{13}C n.m.r. spectra of 2,4-diaminopyrimidines and, as no protons are bonded directly to the carbon atoms of the pyrimidine ring in compound (1), use was made of the longer range C–H couplings observed. Clearly, the three carbon atoms (C-2, -4, and -6) adjacent to ring nitrogens (some bearing amino groups) should experience considerable deshielding and the resonances of all three appear in the range δ_C 162—167. C-6 Is identifiable as giving rise to the resonance at δ_C 166.56, the signal at lowest field in the spectrum of pyrimethamine (1), which is a quartet (^{3}J 5 Hz) owing to three-bond C–H coupling through to the exocyclic C-2″ methyl protons. C-2 and C-4 are far from coupling protons and appear as singlets at δ_C 162.06 and 162.19, although differentiation between these two is not possible in this

Table 1. Proton-coupled ^{13}C n.m.r. spectral data of pyrimethamine (**1**) in $(CD_3)_2SO$

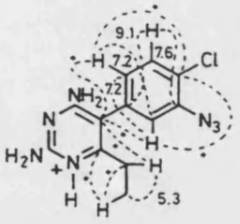
	Free base			Salt (1 × $EtSO_3H$)			
Carbon	δ_C	Mult.	J (Hz)[a]	δ_C	Mult.	J (Hz)[a]	$\Delta\delta$[b]
2	162.06[c]	s		154.88	s		−7.18[c]
	162.19[c]	s					−7.31
4	162.06[c]	s		164.20	s		−2.14[c]
	162.19[c]	s					+2.01
5	105.32	m	*d*	107.26	m	*d*	+1.94
6	166.56	q	5	154.58	m	*d*	−11.98
1′	135.11	t	7.5	133.70	tt	10.8, 3	−1.41
2′/6′	132.64	dd	161.0, 7.1	132.63	dd	162.5, 7.0	−0.01
3′/5′	128.97	dd	165.4, 4.7	129.56	dd	166.3, 4.4	+0.59
4′	131.87	t	7	130.22	t	8.1	−1.65
1″	27.52	tq	126.7, 4.5	23.66	tq	131.0, 4	−3.86
2″	13.76	qt	126.9, 4.5	12.71	qt	128.8, 4.1	−1.05
1‴				45.37	tq	132.9, 4.6	
2‴				9.79	qt	127.4, 4.1	

[a] Coupling constants shown to the nearest integer were estimated graphically, others were taken from the numerical listing generated by the spectrometer. [b] δ (1 × $EtSO_3H$) − δ (free base). [c] Assignment uncertain between the resonances indicated. [d] J < 10 Hz but poorly resolved.

(1)



(2)



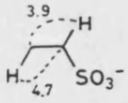


Figure 2. Illustrative ^{13}C–^1H long-range coupling systems observed for the free base of (**1**) and the monoprotonated form of MZP (**2**). Numbers are coupling constants in Hz, * represents poorly resolved coupling with 4 Hz < J < 8 Hz. ^1J Couplings are omitted for clarity

spectrum. The C-5 atom, which is not adjacent to nitrogen, gives a resonance at δ_C 105.32 as a narrow multiplet owing to 3-bond C–H coupling with the protons bonded to C-1″, -2′, and -6′.

When the spectrum of the monoethanesulphonate salt of compound (**1**) was examined, the same methods of assignment were used (Table 1). In this spectrum, however, not only has the coupling from C-1′ to 3′, 5′-H been increased to ^3J 10.8 Hz but the usually very small two-bond aromatic coupling between this carbon and 2′-, 6′-H has been resolved (^2J 3 Hz). The effects of monoprotonation of pyrimethamine (**1**) on the chemical shifts of pyrimidine ring carbons are profound, with a lesser effect on the ethyl group and, as expected owing to the local lack of basic centres, absent in the carbocyclic ring. In the pyrimidine moiety, C-2 and C-6 experience large upfield shifts (*ca.* 7 and *ca.* 12 p.p.m. respectively). These shifts are similar in sign and magnitude to the changes on protonation in the chemical shifts of the *ipso* carbon atoms of anilines, as recently reported by Faure *et al.*;[9] there is also good agreement with the $\Delta\delta$ on protonation observed by Smal *et al.*[10] using specific ^{13}C-labelled trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine]. Thus, it can be inferred that N-1 of the pyrimidine has been protonated, since C-2 and C-6 are both directly bonded to this nitrogen. That protonation has occurred exclusively at N-1 and not at N-3 is shown by the observation that the signal for C-4 does not experience this upfield shift but rather moves slightly downfield.

The ^{13}C n.m.r. spectrum of the free base of the other therapeutic pyrimidine in the study (**2**) is similar to that of pyrimethamine (**1**), with the only exceptions arising in the benzene ring as a result of the additional (azido) substituent at C-3′ (Table 2). Again, no differentiation is possible between the singlet resonances at δ_C 162.01 and 162.30 for C-2 and C-4 in the free base; similarly, the doublets with ^3J coupling at δ_C 136.86 and 136.92 are assigned to C-1′ and C-3′, not necessarily respectively. The contribution of the azido group to the ^{13}C chemical shifts in benzene rings has hitherto not been reported for many compounds. In this case, comparing spectra of compounds (**1**) and (**2**), the effects are approximately +7 p.p.m. (*ipso* carbon; C-3′), −5 p.p.m. (*ortho* carbons; C-2′ and C-4′), +2 p.p.m. (*meta* carbons; C-1′ and C-5′), and −10 p.p.m. (*para* carbon; C-6′). These general values reflect well the nature of the azido group as a pseudohalogen similar to bromine in electronic character and steric factors but are at some quantitative variance with one report[11] for the azido group. When protonated by one equivalent of ethanesulphonic acid, the carbon chemical shifts in the spectrum of MZP (**2**) (Table 2) change very much as do those of pyrimethamine (**1**), again indicating exclusive protonation at N-1. Interestingly, C-1′ experiences a greater upfield shift (some 5 p.p.m.) upon protonation in compound (**2**) than in the case with (**1**) (*ca.* 1.4 p.p.m.).

The analysis and implications of the ^{13}C n.m.r. spectra of the amino analogue (**3**) are more complex, since a basic site attached to the benzene ring can be identified in addition to those on the 2,4-diaminopyrimidine moiety. It is not absolutely clear, *a priori*, whether N-1 or the 3′-amino group would be the site of first protonation, although comparison of the measured pK_a of the conjugate acid of pyrimethamine (**1**) (reported as 7.30[1] and 7.34[5]) with that reported for 2-chloroanilinium ion (2.65),[12] as model compounds for the constituent parts of the amine (**3**), would tend to predict initial protonation at N-1. No prediction is available to select 3′-NH_2 or N-3 (or, indeed, 2-NH_2 or 4-NH_2) as the site of the second protonation.

The spectroscopic data for the free base of compound (**3**) with

Table 2. Proton-coupled ^{13}C n.m.r. spectral data of MZP (2) in $(CD_3)_2SO$

	Free base			Salt (1 × $EtSO_3H$)			
Carbon	δ_C	Mult.	J (Hz)[a]	δ_C	Mult.	J (Hz)[a]	$\Delta\delta$[b]
2	162.01/	s		154.88	s		−7.13/
	162.30[c]	s					−7.42
4	162.01/	s		164.05	s		+2.04
	162.30[c]	s					+1.75
5	104.80	m	*d*	106.67	m	*d*	+1.87
6	166.70	m	*d*	154.7[c]	m	*d*	−12.0
1′	136.86/	d	8.0	131.77	d	9.1	−5.09/
	136.92[c]	d	6				−5.15
2′	128.74	dd	164.2, 7.1	128.41	dd	165.3, 7.2	−0.33
3′	136.86/	d	8.0	137.60	d	7.6	+0.74/
	136.92[c]	d	6.0				+0.68
4′	122.22	m	*d*	124.0[c]	m	*d*	−1.8
5′	130.86	d	166.6	131.31	d	167.7	+0.45
6′	122.75	dd	161.2, 7.4	123.01	dd	162.9, 7.2	+0.26
1″	25.27	tq	127.2, 4.1	23.70	tq	133.1, *d*	−3.87
2″	13.17	qt	126.8, 4.8	12.62	qt	128.4, 5.3	−0.55
1‴				45.31	tq	133.3, 4.7	
2‴				9.70	qt	127.4, 3.9	

[a] Coupling constants shown to the nearest integer were estimated graphically, others were taken from the numerical listing generated by the spectrometer. [b] δ (1 × $EtSO_3H$) − δ (free base). [c] Assignment uncertain between the resonances indicated. [d] J < 10 Hz but poorly resolved. [e] Estimated graphically.

Table 3. Proton-coupled ^{13}C n.m.r. spectral data of compound (3) in $(CD_3)_2SO$

	Free base			Salt (1 × $EtSO_3H$)			
Carbon	δ_C	Mult.	J (Hz)[a]	δ_C	Mult.	J (Hz)[a]	$\Delta\delta_{1-0}$[b]
2	161.96/	s		154.92	s		−7.04/
	162.17[c]	s					−7.25
4	161.96/	s		164.14	s		+2.18/
	162.17[c]	s					+1.97
5	106.29	m	*d*	107.96	m		+1.67
6	166.42	q	4.6	154.40	m	*d*	−12.02
1′	135.59	d	6.8	130.62	d	9.0[e]	−4.97
2′	117.28	dm	167.1, *d*	116.92	dm	157.0,[f] *d*	−0.31
3′	145.04	d	8.6	145.42	d	6.0[f]	+0.38
4′	116.65	m	*d*	117.75	m	*d*	+1.10
5′	129.57	d	162.8	130.03	d	163.4[e]	+0.46
6′	119.19	dd	170.3, 7.7	118.55	dd	160.3,[f] 7.2	−0.64
1″	27.60	tq	127.5, 3.9	23.69	tq	131.1, 4	−3.91
2″	13.53	qt	127.1, 5.0	12.88	qt	128.7, 4	−0.65
1‴				45.35	tq	132.4, 4.6	
2‴				9.72	qt	128.3, 4	

[a] Coupling constants shown to the nearest integer were estimated graphically, others were taken from the numerical listing generated by the spectrometer. [b] δ (1 × $EtSO_3H$) − δ (free base). [c] Assignment uncertain between the resonances indicated. [d] J < 10 Hz but poorly resolved. [e] Decoupled by irradiation of proton resonance centred at δ_H 7.273 (5′-H). [f] J reduced by *ca.* 10 Hz by irradiation as in (*e*).

one equivalent of ethanesulphonic acid are given in Table 3. In the case of the spectrum of the free base, initial assignments were confirmed by a ^1H broadband-decoupled DEPT experiment; C-2′, -5′, -6′, and -2″ appearing as positive peaks, C-1″ as a negative peak, and the remaining (quaternary) carbons giving no signal. Supporting evidence for assignment of the spectrum of the monoethanesulphonate came from selective heteronuclear decoupling. Irradiation at the centre of the 5′-H signal (δ_H 7.273) in the proton spectrum removed the two three-bond couplings of this proton (^3J 9.9 Hz to C-1′ and ^3J 6.0 Hz to C-3′) and, surprisingly in view of the low decoupler power involved, removed the ^1J 163.4 Hz coupling to C-5′. Accidental irradiation of the two other aromatic protons was negligible, resulting only in slight reductions in the magnitudes of ^1J for C-2′ and C-6′. Yet again, the C-2 and C-4 resonances occur as two very close singlets (δ_C 161.96 and 162.17) in the free base. The changes in chemical shift, in the presence of one equivalent of acid, for compound (3) parallel closely those observed for MZP (2) and also correlate well with the $\Delta\delta$ for protonation of pyrimethamine (1). Hence, it can be deduced that the initial protonation of the amine (3) occurs exclusively at N-1. However, when the experiment was carried out in the presence of two equivalents of ethanesulphonic acid, *i.e.* when the crystalline bis(ethanesulphonate) salt[1] of compound (3) is dissolved in DMSO, the resulting ^{13}C n.m.r. spectrum (Table 4) shows only minor to moderate differences from that of the monoethanesulphonate. The maximum deviation in chemical shift of pyrimidine and 6-ethyl carbons betwen the 1 × $EtSO_3H$ and 2 × $EtSO_3H$ spectra is merely 0.23 p.p.m., showing that no change in protonation status has occurred in the pyrimidine

Table 4. Proton-coupled ^{13}C n.m.r. spectral data of compound (3) in $(CD_3)_2SO$

Carbon	Free base δ_C	Salt (2 × $EtSO_3H$) δ_C	Mult.	J (Hz)[a]	$\Delta\delta_{2-0}$[b]	$\Delta\delta_{2-1}$[b]
2	161.96/	154.82	s		−7.14/	−0.10
	162.17[c]				−7.35	
4	161.96/	164.15	s		+2.19/	+0.01
	162.17[c]				+1.98	
5	106.29	107.73	m	*d*	+1.44	−0.23
6	166.42	154.29	m	*d*	−12.13	−0.11
1′	135.59	130.69	d	8.6	−4.90	+0.07
2′	117.28	118.63	dd	160.5, 5	+1.35	+1.66
3′	145.04	143.06	m	*d*	+1.98	−2.36
4′	116.65	119.35	m	*d*	+2.70	+1.60
5′	129.57	130.27	d	164.4	+0.70	+0.24
6′	119.19	118.55	dd	169.7, 5	+1.53	+2.17
1″	27.60	23.60	tq	132.7, *d*	−4.00	−0.09
2″	13.53	12.79	qt	127.0, 5.0	−0.74	−0.09
1‴		45.38	tq	128.8, 4.6		+0.03
2‴		9.54	qt	128.2, 4.2		−0.18

[a] Coupling constants shown to the nearest integer were estimated graphically, others were taken from the numerical listing generated by the spectrometer. [b] $\Delta\delta_{2-0}$: δ (2 × $EtSO_3H$ salt) − δ (free base), $\Delta\delta_{2-1}$: δ (2 × $EtSO_3H$ salt) − δ (1 × $EtSO_3H$ salt). [c] Assignment uncertain between the resonances indicated. [d] J < 10 Hz but poorly resolved.

Table 5. Proton-coupled ^{13}C n.m.r. spectral data of compound (3) in CF_3CO_2D compared with that of free base in $(CD_3)_2SO$

Carbon	MZP (2) Free base δ_C	CF_3CO_2D δ_C	Mult.	J (Hz)[a]	$\Delta\delta_{TFAD-0}$[b]	$\Delta\delta_{TFAD-1}$[b]
2	161.96/	152.12	s		−9.84/	−2.80
	162.17[c]				−10.05	
4	161.96/	160.08	s		−1.88/	−4.06
	162.17[c]				−2.09	
5	106.29	107.36	m	*d*	+1.07	−0.60
6	166.42	159.76	m	*d*	−6.66	+5.36
1′	135.59	127.90/	m	*d*	−7.69/	−2.72/
		128.43[c]	br d	8.0	−7.16	−2.19
2′	117.28	127.25	dd	164.0, 8.0	+10.67	+10.98
3′	145.04	127.90/	m	*d*	−17.14/	−17.52/
		128.43[c]	br d	8.0	−16.61	−16.99
4′	116.65	131.80	m	*d*	+15.15	+14.05
5′	129.57	133.72	d	171.9	+4.15	+3.69
6′	119.19	134.24	dd	165.6, 7.4	+15.05	+15.69
1″	27.60	24.78	tq	132.5, 5.0	−2.82	+1.09
2″	13.53	10.55	qt	130.6, 5.1	−2.98	−2.33

[a] Coupling constants shown to the nearest integer were estimated graphically, others were taken from the numerical listing generated by the spectrometer. [b] $\Delta\delta_{TFAD-0}$: δ(CF_3CO_2D) − δ [free base/$(CD_3)_2SO$], $\Delta\delta_{TFAD-1}$: δ(CF_3CO_2D) − [1 × $EtSO_3H$ salt/$(CD_3)_2SO$]. [c] Assignment uncertain between the resonances indicated. [d] J < 10 Hz but poorly resolved.

ring. Changes in chemical shift of the benzene ring carbons vary from −2.36 p.p.m. for C-3′ to +2.17 p.p.m. for C-6′. Consideration of the signs and relative magnitudes of these $\Delta\delta_{2-1}$, shown in Table 4, and comparison with reported shifts on protonation of aniline in DMSO solution suggest that a small amount of protonation remains at the 3′-NH_2 in solution in an equilibrium which is rapid on the n.m.r. time-scale. When the changes in chemical shift observed here on partial protonation of the 3′-NH_2 (−2.36 p.p.m. for the *ipso* C-3′, +1.66 p.p.m. for the *ortho* carbons, +0.07 and +0.24 p.p.m. for the *meta* carbons, and +2.17 p.p.m. for the *para* carbon) are compared with those reported[9] for full protonation of aniline in the same solvent (*ipso* −16.7 p.p.m., *ortho* + 9.4 p.p.m., *meta* + 1.0 p.p.m., and *para* +12.3 p.p.m.), it can be inferred that the 3′-amino group is *ca.* 16% protonated. Hence, the 3′-ammonium function which is present in the solid state dissociates to the extent of *ca.* 84% upon dissolution in DMSO.

Since compound (3) is largely only monoprotonated in the presence of two equivalents of ethanesulphonic acid, it was necessary to make use of much higher acid concentrations to examine the spectra of multiprotonated species from compound (3). The proton-coupled ^{13}C n.m.r. spectral data of amine (3) in neat deuteriotrifluoroacetic acid are listed in Table 5. This spectrum, although slightly less well resolved than the others, is still capable of full assignment except that the resonances for C-1′ and C-3′ could not be distinguished between signals at δ_C 127.90 and 128.43. Clearly, the 3′-amino group is fully ionised in this medium and corresponding characteristic chemical-shift differences between the spectrum of the free base of (3) in DMSO (and, incidentally, the monoethanesulphonate) and that in CF_3CO_2D solution are observed. The $\Delta\delta_{TFAD-1}$ values for the benzene-ring protons are: *ipso* carbon (C-3′) *ca.* −17 p.p.m., *ortho* carbon (C-2′) + 10.98 p.p.m., *ortho* carbon (C-4′) + 14.05 p.p.m., *meta* carbon (C-1′) *ca.* −2.5 p.p.m., *meta* carbon (C-5′) + 3.69 p.p.m., *para* carbon (C-6′) + 15.69 p.p.m. These data are in good agreement with the changes in chemical shift on

Table 6. ^{1}H N.m.r. spectral data of compound (3) ethanesulphonate salts in $(CD_3)_2SO$. Only C–H signals are shown

	Salt (1 × $EtSO_3H$)			Salt (2 × $EtSO_3H$)		
Proton	δ_H	Mult.	J (Hz)	δ_H	Mult.	J (Hz)
2′-H	6.65	d	2.0	6.69	d	2.0
5′-H	7.27	d	8.1	7.32	d	8.1
6′-H	6.40	dd	8.1, 2.0	6.47	dd	8.1, 2.0
1″-H_2	2.23	br q	7.6	2.22	br q	7.6
2″-H_3	1.05	br t	7.6	1.04	t	7.6
1‴-H_2	2.57	q	7.4	2.53	q	7.4
2‴-H_3	1.12	t	7.4	1.09	t	7.4

Table 7. ^{1}H N.m.r. spectral data of compound (3) in $(CD_3)_2SO$ and in CF_3CO_2D. Only C–H signals are shown

	Free base [$(CD_3)_2SO$]			CF_3CO_2D		
Proton	δ_H	Mult.	J (Hz)	δ_H	Mult.	J (Hz)
2′-H	6.65	d	1.5	7.84	br d	1.5[a]
5′-H	7.25	d	8.0	7.93	d	8.3
6′-H	6.38	dd	8.0, 1.5	7.61	dd	8.3, 1.5
1″-H_2	2.18	q	7.5	2.60	q	7.5
2″-H_3	1.00	t	7.5	1.29	t	7.5

[a] Estimated graphically.

protonation of aniline reported[9] by Faure *et al.* (see above), subject to the effects of change of solvent (predicted to be <3 p.p.m.).[9] Examination of the resonances from the pyrimidine carbons in CF_3CO_2D (Table 5) and comparison with the mono-(N-1)-protonated species shows some additional protonation on this heterocycle or its amino substituents. More detailed examination of chemical-shift differences between amine (3) in its monoprotonated form in DMSO and in trifluoroacetic acid (TFA) shows upfield shifts for C-2 and C-4 and a downfield shift for C-6 (Table 5), indicative of protonation at N-3. The magnitudes of the $\Delta\delta$ values involved, when compared with the magnitudes of the effects of protonation at N-1, suggest however that this protonation is incomplete even in this highly acidic medium.

Proton n.m.r. spectra were also recorded for the solutions of amine (3) and acids as detailed above for the ^{13}C n.m.r. spectra; the data are recorded in Tables 6 and 7. The resonances for the C–H protons are very similar in the spectra of amine in DMSO in the presence of 0, 1, and 2 equiv. of ethanesulphonic acid, confirming that only slight protonation of the 3′-NH_2 group occurs under these conditions. In neat TFA, full protonation at 3′-NH_2 is reported by downfield shifts of *ca.* 1.1 p.p.m. for 2′-H and 6′-H (*ortho* and *para* respectively to the amino group) and of 0.66 p.p.m. for 5′-H (*meta*).

In conclusion, it can be seen from the above data that the first protonation of all three 2,4-diaminopyrimidines studied takes place on N-1 in DMSO solution, as predicted by *X*-ray crystallographic work.[6] This protonation is complete in the presence of only 1 equiv. of ethanesulphonic acid, indicating a relatively high basicity of N-1 in these molecules, which is consistent with values for the overall values of pK_a of the conjugate acids determined by conventional techniques [7.3 for (1),[1,5] 7.2 for (2),[1] and 7.5 for (3)[13]]. The second protonation of amine (3) has been shown to take place extensively only at very high acid strengths and then at 3′-NH_2. This conclusion allows the rationalisation of the observation that amine (3) is an inhibitor of DHFR [although weaker than (1) or (2)][1] according to the classical requirements for protonation at N-1, but at no other sites, at physiological pH for binding to the active site of the enzyme.

Experimental

Spectroscopy.—N.m.r. spectra were obtained at 100.8 MHz (^{13}C) and at 400 MHz (^{1}H) with a Bruker WH400 spectrometer. ^{13}C Spectra were recorded at natural abundance without decoupling from protons except that selective irradiation of the proton resonance at δ_H 7.273 was used in one ^{13}C experiment using the monoethanesulphonate of compound (3) in order to confirm assignment of certain ^{13}C resonances. All chemical shifts are referenced to tetramethylsilane as internal standard. Spectra were recorded at 300 ± 2 K at *ca.* 10% w/v concentration.

Materials.—Pyrimethamine (1) was a kind gift of The Wellcome Foundation Ltd., Dartford, Kent. The amino analogue (3) was prepared by nitration (HNO_3–H_2SO_4) and subsequent reduction ($SnCl_2$) of compound (1); diazotisation (aq. HCl–$NaNO_2$) and treatment with sodium azide furnished the free base of compound (2) as previously reported.[1] The crystalline monoethanesulphonate salts of compounds (1) and (2) and bis(ethanesulphonate) salt of compound (3) were prepared by treatment of the free bases with aqueous ethanesulphonic acid followed by recrystallisation.[1] The spectroscopic sample for the monoethanesulphonate salt of compound (3) was prepared by dissolution of equimolar amounts of compound (3) (free base) and ethanesulphonic acid in $(CD_3)_2SO$; correct proportions were checked by integration of the proton n.m.r. spectrum.

Acknowledgements

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Structural Studies on Bio-Active Compounds. Part 7. The Design and Synthesis of α-Substituted Serines as Prospective Inhibitors of Serine Hydroxymethyltransferase

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Structural Studies on Bioactive Compounds. Part 7.[1] The Design and Synthesis of α-Substituted Serines as Prospective Inhibitors of Serine Hydroxymethyltransferase

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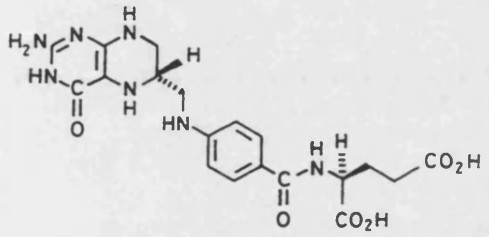
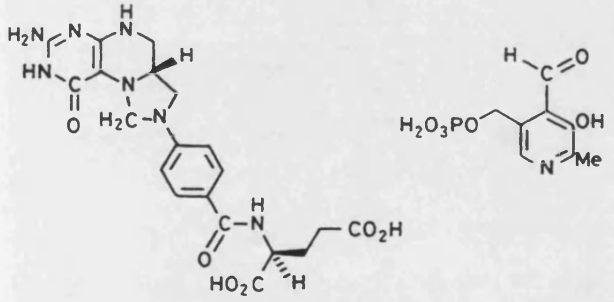
A short series of α-substituted analogues of serine, designed as enzyme-activated, irreversible inhibitors of serine hydroxymethyltransferase, has been prepared. (±)-α-Allyl- and (±)-α-prop-2-ynyl-serine were synthesised by appropriate alkylation of the anion derived from ethyl acetamidocyanoacetate, followed by selective reduction of the ester function and hydrolysis of protecting groups. Acetoxymethylation of the anion derived from methyl 2-(benzylideneamino)but-2-enoate gave (±)-α-vinylserine after deprotection. These novel analogues of serine were largely inactive as inhibitors of the enzyme, except that (±)-α-vinylserine showed weak competitive inhibition.

Serine hydroxymethyltransferase (SHMT, EC 2.1.2.1) catalyses the conversion of L-serine (**4**) to glycine (**5**) in the presence of tetrahydrofolic acid (FH_4) (**1**), generating a one-carbon unit in



(**1**)



(**2**) (**3**)

the form of N^5,N^{10}-methylenetetrahydrofolate (FH_4-CH_2) (**2**).[2] This one-carbon unit may then be used either for the biosynthesis of purines and pyrimidines or for other anabolic processes. The enzyme is a tetramer with each subunit having a coenzyme, pyridoxal phosphate (PLP) (**3**).[3] The enzyme SHMT appears to have an essential role in rapidly proliferating tumour cells which have a high requirement for L-serine.[4] In these cells, it has elevated activity in channelling one-carbon units into *de novo* nucleoside synthesis.[5] If SHMT could be inhibited, then it may be possible to inhibit the growth of these cells as a strategy for cancer chemotherapy.

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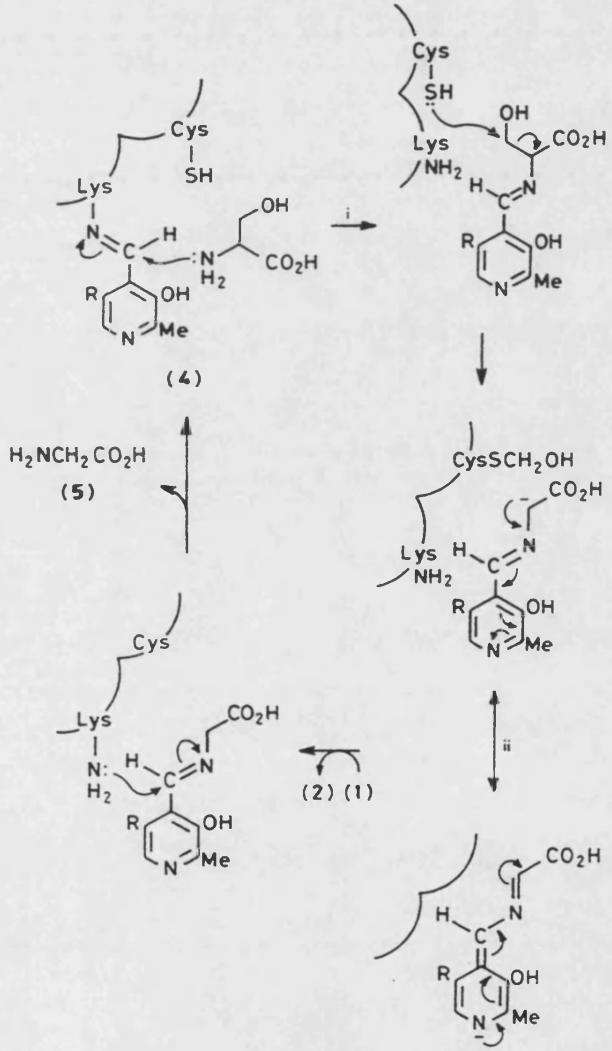
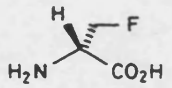


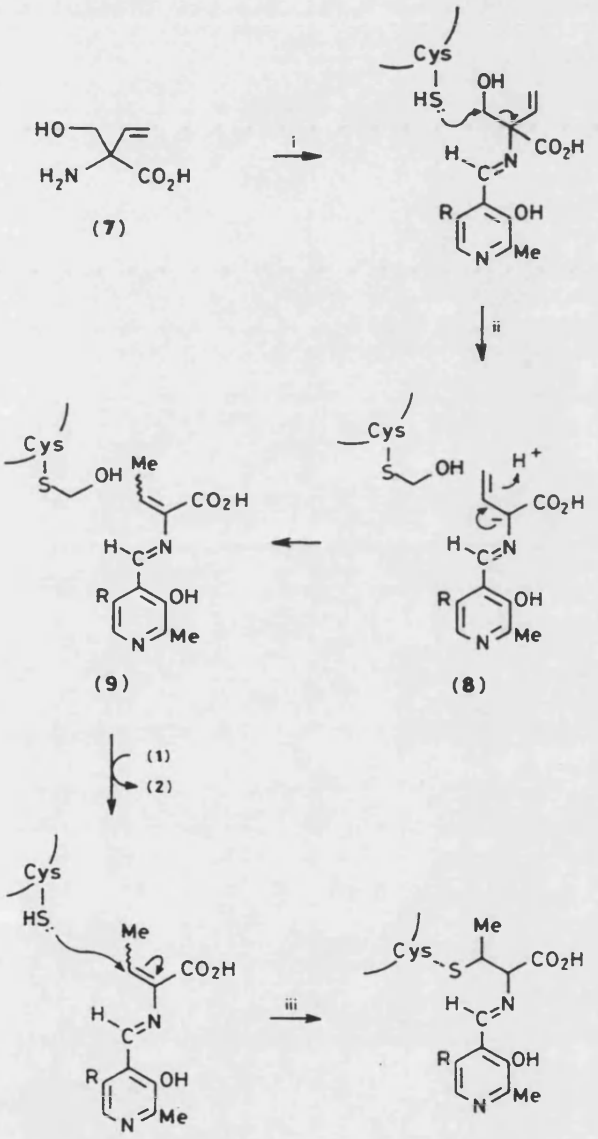
Scheme 1. Probable mechanism of serine hydroxymethyltransferase: i, Transimination; ii, delocalisation of negative charge. R = CH_2O-PO_3H^-

L-Serine (4) binds to the active site of the enzyme by a transimination reaction, as shown in Scheme 1, and is cleaved by a reverse-aldol reaction to a stabilised carbanion. Protonation of the carbanion followed by a reversal of the initial transimination produces glycine (5) and FH_4–CH_2 (2). D-Fluoroalanine (6) (a product analogue) has been shown[6] to



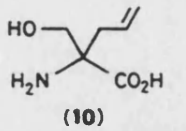
(6)

be an enzyme-activated irreversible inhibitor of this enzyme. The aim of the present work was to prepare a more effective inhibitor of this type. α-Vinylserine (7) was designed as an irreversible inhibitor with a proposed mechanism of action, shown in Scheme 2, analogous to the mechanism of inhibition of

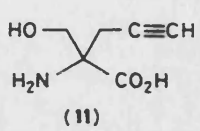


Scheme 2. Designed mechanism for the inhibition of serine hydroxymethyltransferase by (7): i, binding to the active site of the enzyme; ii, reverse aldol reaction; iii, conjugate addition. R = $CH_2OPO_3H^-$

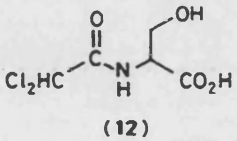
the PLP-dependent enzymes by α,β-unsaturated amino acids. The reverse aldol product (8) may be reprotonated at the terminal carbon giving an electrophilic α,β-unsaturated acid (9) as a soft centre to react with the thiol[7] at the active site, irreversibly inhibiting the enzyme. α-Vinyl amino acids have previously been found to be effective inhibitors of a range of PLP dependent enzymes.[8] Two other serine analogues, α-allylserine (10) and α-prop-2-ynylserine (11), with unsaturated substituents may also be proposed as irreversible inhibitors of SHMT. *N*-(Dichloroacetyl)serine (12) has been reported to be an active agent against experimental rodent tumours[9,10] and was also evaluated in this study.



(10)



(11)

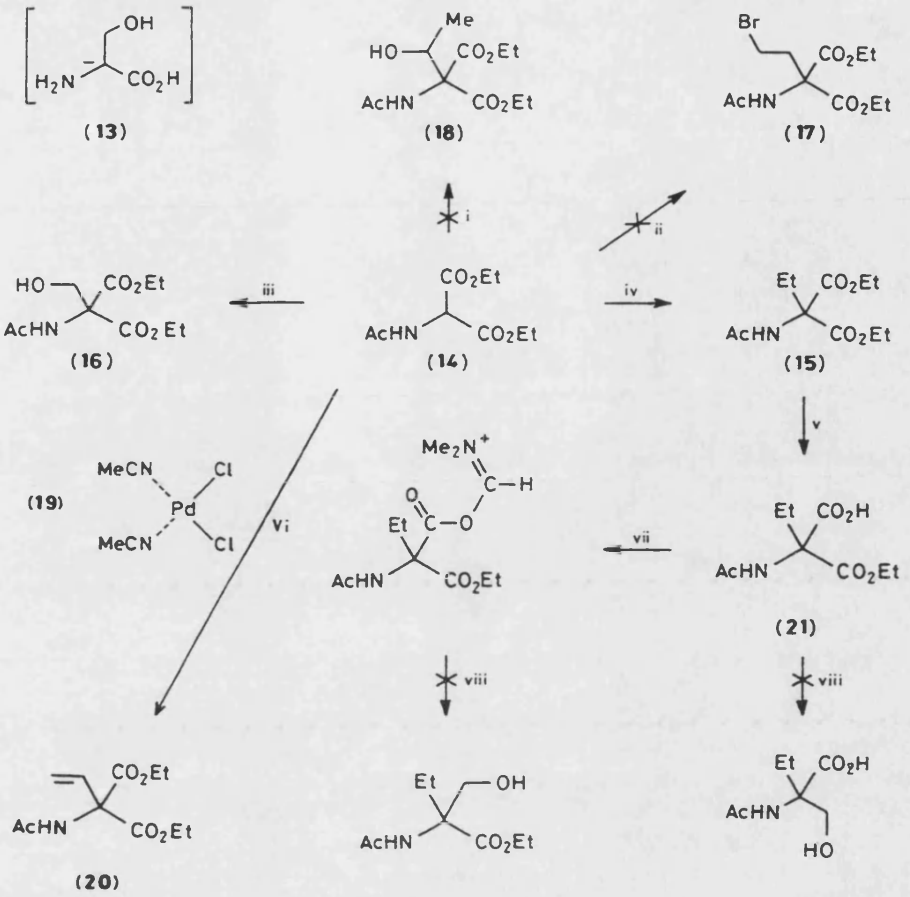


(12)

Initial approaches to the introduction of substituents at the α-carbon of serine involved the use of diethyl acetamidomalonate (14) as a synthon for the serine α-anion (13) (Scheme 3). In a model sequence, the sodium derivative of (14) was cleanly alkylated in ethanolic solution using bromoethane to give the diester (15). The malonate (14) also reacted with aqueous formaldehyde in the presence of sodium hydroxide to afford the hydroxymethyl compound (16). All attempts to prepare the 2-bromoethyl compound (17), for possible subsequent elimination of hydrogen bromide to give a vinyl compound, were unsuccessful. Similarly, diethyl acetamidomalonate did not react with acetaldehyde to give the 1-hydroxyethyl derivative (18). Heyashi has reported[11] the attachment of a vinyl group to dimethyl malonate by treatment of the anion of the diester with ethylene in the presence of a stoichiometric amount of the square planar complex bis(acetonitrile)palladium(II) chloride (19). The synthetic scope of this potentially useful reaction is extended by our observation that ethylene reacts with diethyl acetamidosodiomalonate in the presence of (19) to afford the α-vinyl compound (20) after work-up in air. This material was, however, found to be unstable and thus to be unsuitable for the proposed synthetic route to α-vinylserine (7).

One ester group of the model malonate diester (15) was hydrolysed selectively with ethanolic potassium hydroxide, to give the monoester (21). Selective reduction of either the remaining ester or of the carboxylic acid should then give a protected α-ethylserine. The selective reduction of the ester group in ethyl acetamidocyanoacetate has been reported[12] to be effected by sodium borohydride but this reagent was without effect on the substrate (21), possibly owing to prior ionisation of the carboxylic acid. Likewise, the selective reduction of the carboxylic acid of (21) by sodium borohydride was unsuccessful after activation with oxalyl chloride–*N*,*N*-dimethylformamide.[13]

Scheme 4 shows the route employed for the synthesis of (7). (±)-Threonine (22), a readily available starting material with most of the required carbon skeleton, was converted to its methyl ester hydrochloride (23) in the usual way by treatment with boiling methanolic hydrogen chloride. Elimination of the hydroxy group could not be achieved directly, so phosphorus pentachloride was used to replace the hydroxy group, giving the



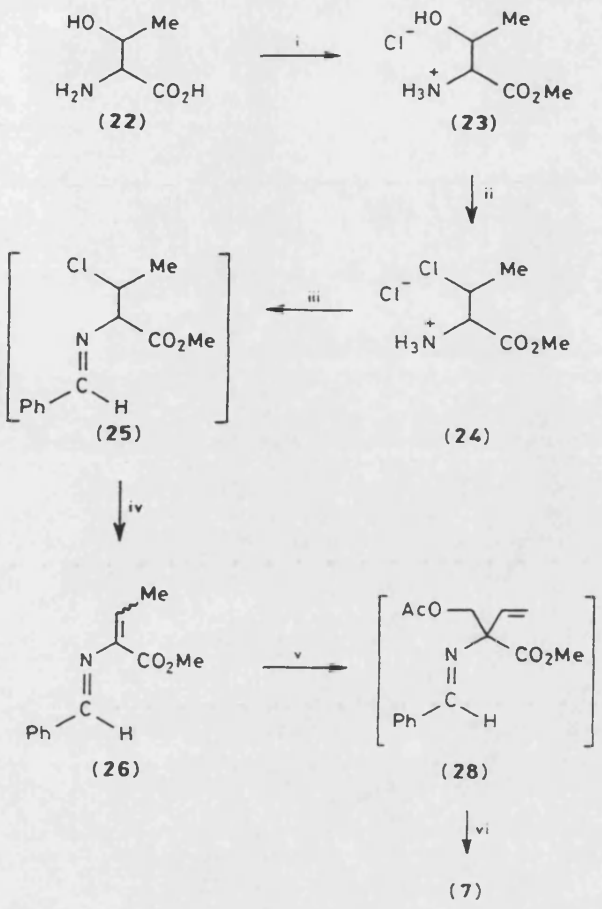
Scheme 3. Attempted syntheses of α-substituted serines using (**14**) as a synthon for (**13**): i, MeCHO–base; ii, $BrCH_2CH_2Br$–base; iii, HCHO–aq.NaOH; iv, EtBr–NaOEt–EtOH; v, KOH–EtOH; vi, NaH–THF–C_2H_4–(**19**); vii, oxalyl chloride–DMF; viii, $NaBH_4$

chloro compound (**24**) in which azirane formation is suppressed by protonation of the primary amine. Concomitant activation of the α-proton and protection was most conveniently effected by condensation of (**24**) with benzaldehyde to give the imine (**25**).

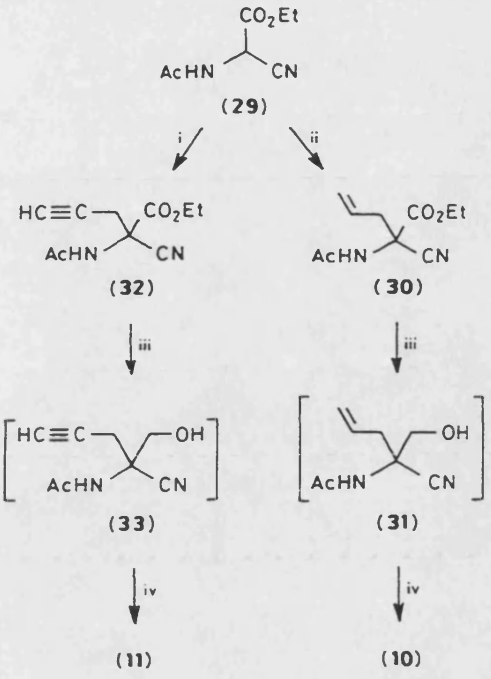
1,5-Diazabicyclo[5.4.0]undec-7-ene (DBU), a non-nucleophilic bicyclic amidine base, was used to eliminate hydrogen chloride to give methyl 2-(benzylideneamino)but-2-enoate (**26**). This dehydroamino acid represents the carbon framework for the synthesis of α-vinyl substituted amino acids by alkylation of the α-carbon.[14] The lithiated compound was prepared using lithium hexamethyldisilazide and this anion was smoothly acetoxymethylated with chloromethyl acetate (**27**) in the presence of hexamethylphosphoric triamide, providing a convenient method of introducing a protected hydroxymethyl group into the compound. Direct hydroxymethylation with formaldehyde was not possible in the non-aqueous environment required to prepare the anion; chloromethyl acetate was selected as a suitable synthon consistent with the required conditions. The chloromethyl acetate (**27**) had been previously prepared from acetyl chloride and paraformaldehyde.[15] The protecting groups were then removed from (**28**) in two stages by hydrolysis with aqueous acid and the (±)-α-vinylserine (**7**) was isolated after neutralisation with aqueous ammonia.

Selective reductions were, however, employed in the syntheses of α-allyl- and α-prop-2-ynyl-serine (**10**) and (**11**), as shown in Scheme 5. The sodium derivative of ethyl acetamidocyanoacetate (**29**) reacted smoothly with allyl bromide to give the substituted ethyl pentenoate (**30**). In this case, reduction with the more active lithium borohydride proved to be more successful than the attempts using the sodium analogue (*vide supra*). However, the immediate reduction product (**31**) could not be isolated from the resulting borate complexes, so it was hydrolysed with 6M-hydrochloric acid. Neutralisation with aqueous ammonia gave α-allylserine (**10**) in moderate yield. Ethyl acetamido(cyano)sodioacetate, prepared in absolute ethanol, failed to react with prop-2-ynyl bromide. However, the use of toluene, a less solvating solvent, facilitated the alkylation. The resulting substituted ethyl pentynoate (**32**) was then similarly reduced with lithium borohydride and the intermediate (**33**) was hydrolysed with 6M-hydrochloric acid to give α-prop-2-ynylserine (**11**) after cation exchange column chromatography using Dowex 50-X8.

The novel α-substituted serine analogues (**7**), (**10**), and (**11**) and the *N*-dichloroacetyl compound (**12**) were initially tested against K562 human erythroid leukaemia total cell SHMT using the assay method of Taylor and Weissbach.[16] The production of [^{14}C]formaldehyde from L-[3-^{14}C]serine proceeded in a linear manner for 20 min, with total protein concentrations of up to 0.3 mg protein per 0.2 ml incubation. The activity of SHMT for the K562 total cell enzyme was determined by measuring the rate of formation of [^{14}C]formaldehyde during



Scheme 4. Synthesis of (7). i, MeOH–HCl; ii, PCl_5–CH_2Cl_2; iii, PhCHO–$MgSO_4$–CH_2Cl_2–Et_3N; iv, DBU–CH_2Cl_2; v, Li^+-$N(SiMe_3)_2$–$ClCH_2OAc$ (27)–THF–HMPA; vi, aq. HCl



Scheme 5. Synthesis of (10) and (11): i, $HCCCH_2Br$–NaH–PhMe; ii, H_2CCHCH_2Br–NaOEt–EtOH; iii, $LiBH_4$–THF; iv, aq. HCl

20 min. Duplicate determinations were consistent within 5%. All four compounds had little inhibitory effect on the enzyme activity, at concentrations of 0.25—4 mmol l^{-1}.

To establish whether or not the substituted serines (7), and (10)—(12) are effective inhibitors of total cell SHMT, and to classify the type of any inhibition, the kinetics of the effects of each compound were evalulated. The rate of reaction was determined in the presence of serine (0.25—3 mM) and the test amino acid (2.5—10 mM). Only α-vinylserine deviated from the control in this assay. Lineweaver–Burk plots (Figure) show this compound to be a competitive inhibitor of total K562 cell SHMT (K_i 15.2 mmol l^{-1}). To investigate the effects of the α-substituted serine analogues on whole cells, the growth inhibitory effects of these compounds against the GM0621 and K562 leukaemia cell lines was determined. At concentrations < 10 mM, the serine analogues showed no toxicity. The activation of the amino acids (7), (10), and (11) as inhibitors of SHMT depends on the capability of the enzyme to accept substrate analogues with substituents on the α-carbon of serine. While it is known [17] that the enzyme will accept β-substituted serines, the ability of the enzyme to catalyse the removal of the CH_2OH group from α-substituted serines has yet to be fully explored. However, it has been reported [18] that L-α-methylserine and α-hydroxymethylserine (34) are substrates, although the latter, (34), has K_m 20 mol l^{-1} (the K_m for the natural substrate, serine, is 4.3 mol l^{-1}). This suggests that the active site of the

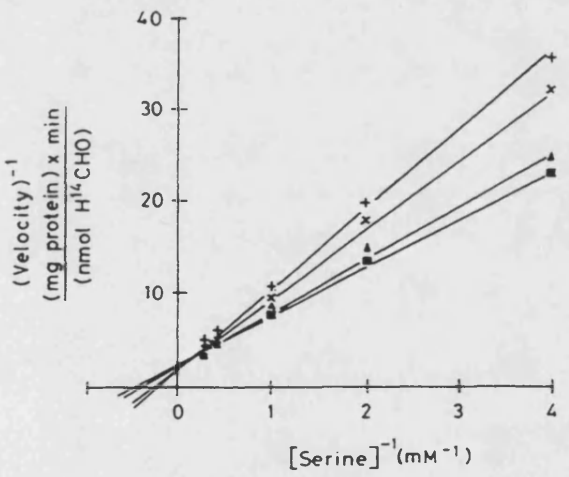
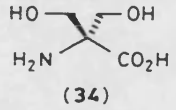


Figure. Lineweaver–Burk plots for the kinetics of inhibition of serine hydroxymethyltransferase by (7). Concentrations of (7): + 10; × 5; ▲ 2.5 mol l^{-1}; ■ Control (0 mmol l^{-1})

enzyme should be able to accept α-substituted serines of similar steric bulk. The longer chain unsaturated analogues (10) and (11) caused no inhibition of the enzyme from the K562 cells.



(34)

This may suggest that either these compounds have substituents that are too bulky for the enzyme to accept or that removal of the β-proton by the enzyme, a prerequisite for the activation of

these compounds as irreversible inhibitors, does not occur. (±)-α-Vinylserine was shown to be an inhibitor (K_i 15.2 mol l^{-1}). This inhibition was, however, shown to be reversible, in contrast to the designed mechanism. The nature of the binding of (7) to the enzyme is not clear, although it appears to compete with serine for the binding site. Further investigations with partially purified enzymes will be conducted to reveal the true nature of this interaction.

Experimental

I.r. spectra were recorded on Pye-Unicam SP200 or Perkin-Elmer 1310 spectrometer as potassium bromide discs unless otherwise stated. ^{1}H N.m.r. spectra were recorded at 60 MHz using a Varian EM360A spectrometer and at 200 MHz with a Bruker AM200 instrument and are referenced to tetramethylsilane as the internal standard. Mass spectra were obtained using a VG Micromass 12B single focussing mass spectrometer in the electron-impact mode. M.p.s are uncorrected. Compounds are racemic unless otherwise stated.

Ethyl 2-Acetamido-2-(ethoxycarbonyl)but-3-enoate (**20**).—Dry tetrahydrofuran (38.5 ml), containing bis(acetonitrile)-palladium(II) dichloride (**19**) (500 mg, 1.9 mmol) was saturated with ethylene at −70 °C. Triethylamine (0.54 ml, 3.8 mmol) was added, followed after 15 min by diethyl acetamido(sodio)-malonate (1.9 mmol) in dry tetrahydrofuran (10 ml). The mixture was stirred at −5 °C for 1 h and warmed to ambient temperature during 70 min. After the palladium(0) had been removed by filtration, rapid chromatography of the evaporation residue (silica gel, ethyl acetate) yielded *ethyl 2-acetamido-2-(ethoxycarbonyl)but-3-enoate* (290 mg, 62%) as an unstable colourless oil, $\nu_{max.}$ 1 520, 1 640, and 1 740 cm^{-1}; $\delta(CDCl_3)$ 1.25 (6 H, t, *J* 7 Hz, CH_2*Me*), 2.10 (3 H, s, COMe), 4.30 (4 H, q, *J* 7 Hz CH_2Me), 5.25 (1 H, d, *J* 17 Hz, 4-H), 5.30 (1 H, d, *J* 10 Hz, 4-H), 6.55 (1 H, dd, *J* 17, 10 Hz, 3-H), and 7.1 (1 H, s, NH).

Chloromethyl Acetate (**27**).—Acetyl chloride (25.9 g, 33 mmol), paraformaldehyde (9.0 g, 33 mmol HCHO) and anhydrous zinc chloride (*ca.* 100 mg) were warmed together to 50 °C. After a short induction period, the heat of reaction caused the solution to boil gently for 3 h. The mixture was filtered through basic alumina to give a yellow liquid (33.0 g). N.m.r. spectroscopy revealed this to comprise 92% chloromethyl acetate and 8% bis(chloromethyl) ether (yield of ester 85%). This material was used for later experiments without further purification and had $\nu_{max.}$ 1 760 cm^{-1}; $\delta(CDCl_3)$ 2.10 (3 H. s, Me) and 5.60 (2 H, s. CH_2). The contaminating ether had $\delta(CDCl_3)$ 5.55 (s, 2 × CH_2).

Methyl 2-Amino-3-chlorobutanoate Hydrochloride (**24**).—Phosphorus pentachloride (6.35 g, 30 mmol) was added to a suspension of threonine methyl ester hydrochloride (**23**) (5.0 g, 29 mmol) in dichloromethane (260 ml) during 1 h and the resulting solution was stirred at ambient temperature for 3 h then cooled to 0 °C for 16 h. The white crystals were collected by filtration, washed with diethyl ether and light petroleum (b.p. 60—80 °C), and recrystallised from acetonitrile to afford methyl 2-amino-3-chlorobutanoate hydrochloride (3.7 g, 68%) as white crystals, m.p. 169—171 °C (lit.,[19] 169—172 °C), $\nu_{max.}$ 1 500, 1 600, 1 740, and 2 500—3 000 cm^{-1}; $\delta(D_2O)$ 1.65 (3 H, d, *J* 7 Hz, 4-H_3), 3.95 (3 H, s, OMe), 4.60 (1 H, d, *J* 3 Hz, 2-H), and 4.8 (4 H, m, N^+H_3 and 3-H).

Methyl 2-(Benzylideneamino)but-2-enoate (**26**).—Benzaldehyde (4.5 g, 44 mmol) was added to a well-stirred slurry of methyl 2-amino-3-chlorobutanoate hydrochloride (**24**) (8.3 g, 44 mmol) in dichloromethane (80 ml) at 5 °C, followed by triethylamine (6.15 ml, 44 mmol) and dried magnesium sulphate (4.0 g). The resulting suspension was stirred at ambient temperature for 21 h before water (100 ml) was added. The aqueous layer was extracted with dichloromethane (3 × 50 ml) and the organic solutions were combined, washed with saturated aqueous sodium chloride, and dried ($MgSO_4$). Evaporation of the solvent under reduced pressure afforded crude methyl 2-(benzylideneamino)-3-chlorobutanoate (11.1 g), $\delta(CDCl_3)$ 1.55 (3 H, d, *J* 7 Hz, 4-H_3), 3.75 (3 H, s, OMe), 4.10 (1 H, d, *J* 7 Hz, 2-H), 4.65 (1 H, quintet, *J* 7 Hz, 3-H), 7.3—7.9 (5 H, m, ArH), and 8.35 (1 H, s, PhC*H*). 1,5-Diazabicyclo[5.4.0]undec-5-ene (6.5 ml) was added dropwise during 5 min to the above ester in dichloromethane (80 ml) at 5 °C. After 3 h, water (100 ml) was added and the organic layer was separated and dried ($MgSO_4$). Evaporation of the solvent under reduced pressure gave an oil which, in hexane–diethyl ether (10:1), was filtered through neutral alumina (20 g) to give, after evaporation of the solvents, methyl 2-(benzylideneamino)but-2-enoate (8.4 g, 94%) as a colourless oil, $\nu_{max.}$ (liquid film) 1 580, 1 600, 1 640, and 1 715 cm^{-1}; $\delta(CDCl_3)$ 1.90 [2.1 H, d, *J* 7 Hz, 4-H_3 (*E*)], 2.05 [0.9 H, d, *J* 7 Hz, 4-H_3 (*Z*)], 3.70 [2.1 H, s, OMe (*E*)], 3.75 [0.9 H, s, OMe (*Z*)], 5.85 [0.3 H, q, *J* 7 Hz, 3-H (*Z*)], 6.50 [0.7 H, q, *J* 7 Hz, 3-H (*E*)], 7.4 (3 H, m) and 7.8 (2 H, m) (Ar-H), 8.25 [0.3 H, s, ArC*H* (*Z*)], and 8.45 [0.7 H, s, ArC*H* (*E*)]; *m/z* 203 (M^+) and 143 (100%).

2-Amino-2-(hydroxymethyl)but-3-enoic Acid (*α-Vinylserine*) (**7**).—Lithium hexamethyldisilazide was prepared by the addition of butyl-lithium (8.0 ml, 12.3 mmol; 1.55M in hexane) to hexamethyldisilazane (2.0 g, 12.3 mmol) in tetrahydrofuran (freshly distilled from calcium hydride; 5 ml) at −70 °C under nitrogen. Hexamethylphosphoric triamide (7 ml) and methyl 2-(benzylideneamino)but-2-enoate (**26**) (2.3 g, 11.2 mmol) were then added, followed by chloromethyl acetate (**27**) (1.35 g, 12.3 mmol) in dry tetrahydrofuran (10 ml). The mixture was stirred for 1 h at −70 °C and for 2 h at ambient temperature before being diluted with saturated aqueous ammonium chloride and extracted with diethyl ether (3 × 50 ml). The combined organic extracts were washed with water (2 × 50 ml) and with saturated aqueous sodium chloride (20 ml) and dried ($MgSO_4$). Evaporation of the solvent under reduced pressure gave a yellow oil which was treated with hydrochloric acid (2M; 40 ml) for 2 min. The resulting solution was washed with diethyl ether (2 × 50 ml) and with chloroform (2 × 50 ml). Hydrochloric acid (6M; 20 ml) was added and the whole was boiled under reflux for 2 h before being washed with dichloromethane (2 × 10 ml) and treated with activated charcoal (100 mg). The filtered solution was concentrated to 10 ml under reduced pressure and neutralised by the addition of aqueous ammonia. Hot ethanol (25 ml) was added and, upon cooling, white crystals were deposited. Recrystallisation of these from aqueous ethanol yielded *2-amino-2-(hydroxymethyl)but-3-enoic acid* (170 mg, 11.6%) as fine white needles m.p. 173—175 °C (decomp.) (Found: C, 45.5; H, 7.1; N, 10.8. $C_5H_9NO_3$ requires C, 45.8; H, 6.9; N, 10.7%); $\nu_{max.}$ (Nujol) 1 510, 1 590, 1 610, and 1 640 cm^{-1}; $\delta(D_2O)$ 3.80 (1 H, d, *J* 12 Hz) and 4.00 (1 H, d, *J* 12 Hz) (CH_2OH), 5.35 (1 H, d, *J* 18 Hz, 4-H), 5.44 (1 H, d, *J* 12 Hz, 4-H), and 6.02 (1 H, dd, *J* 12, 18 Hz, 3-H); *m/z* 100 ($M^+ - CH_2OH$) and 54 (100%).

Ethyl 2-Acetamido-2-cyanopent-4-enoate (**30**).—Sodium (1.15 g, 50 mmol) was dissolved in dry ethanol (30 ml) and ethyl acetamidocyanoacetate (**29**) (8.5 g, 50 mmol) and 3-bromopropene (6.5 g, 50 mmol) were added. The mixture was boiled under reflux for 16 h before the solvent was evaporated under reduced pressure. The residue was washed with water (30 ml) and recrystallised from aqueous ethanol to give ethyl 2-acetamido-2-cyanopent-4-enoate (4.1 g, 39%) as a white solid,

m.p. 83—85 °C (lit.,[20] 86—88.5 °C), $\nu_{max.}$ 1 515, 1 650, and 1 740 cm^{-1}; δ($CDCl_3$) 1.35 (3 H, t, *J* 7 Hz, CH_2Me), 2.05 (3 H, s, COMe), 2.80 (2 H, d, *J* 6 Hz, 3-H_2), 4.30 (2 H, q, *J* 7 Hz, CH_2Me), 5.2—5.9 (3 H, m, 4-H and 5-H_2), and 7.5 (1 H, s, NH); *m/z* 211 (M^+ + 1) and 95 (100%).

2-Amino-2-(hydroxymethyl)pent-4-enoic Acid (*α-allylserine*) (**10**).—Lithium borohydride (360 mg, 16 mmol) in dry tetrahydrofuran (10 ml) was added dropwise during 5 min to a suspension of ethyl 2-acetamido-2-cyanopent-4-enoate (**30**) (3.5 g, 16 mmol) in dry tetrahydrofuran (50 ml) and the mixture was boiled under reflux for 3 h before being diluted with methanol (50 ml) and acidified with hydrochloric acid (6M; 2 ml). Evaporation of the solvents under reduced pressure gave a yellow gum which, in hydrochloric acid (6M; 20 ml), was boiled under reflux for 2 h. Evaporation of the solvent and the excess of reagent under reduced pressure followed by neutralisation with aqueous ammonia gave a white powder from which *2-amino-2-(hydroxymethyl)pent-4-enoic acid* (460 mg, 20%) was isolated by recrystallisation from aqueous ethanol. The white crystals had m.p. 215—220 °C (decomp.) (Found: C, 49.6; H, 7.7; N, 9.7. $C_6H_{11}NO_3$ requires C, 49.6; H, 7.6; N, 9.6%); $\nu_{max.}$ 1 510, 1 610, 1 640, 2 500—3 200, and 3 450 cm^{-1}; δ(D_2O) 2.40 (1 H, dd, *J* 14.6, 9.3 Hz) and 2.60 (1 H, dd, *J* 14.6, 6.3 Hz) (3-H_2), 3.71 (1 H, dd, *J* 11.2 Hz) and 3.95 (1 H, dd, *J* 11.2 Hz) (CH_2OH), 5.27 (1 H, d, *J* 12.2 Hz, 5-H), 5.28 (1 H, d, *J* 15.1 Hz, 5-H), and 5.7 (1 H, m, 4-H); *m/z* 126 ($M^+ - CH_2OH$) and 114 (100%).

Ethyl 2-Acetamido-2-cyanopent-4-ynoate (**32**).—Ethyl acetamidocyanoacetate (**29**) (17.0 g, 100 mmol) was added to a suspension of sodium hydride (50% in oil; 4.8 g, 100 mmol) in dry toluene (100 ml), followed by 3-bromopropyne (70% in toluene, 14.8 g, 180 mmol) and the mixture was boiled under reflux for 3 h. Ethyl acetate (20 ml) was added, followed by water (50 ml) and diethyl ether (50 ml). The combined organic solutions were concentrated under reduced pressure and, when allowed to stand, deposited light brown crystals. Recrystallisation of these from ethanol–light petroleum (b.p. 60—80 °C) furnished *ethyl 2-acetamido-2-cyanopent-4-ynoate* (5.2 g, 25%) as very pale buff crystals m.p. 87—88 °C; $\nu_{max.}$ 1 660, 1 760, and 2 500—3 500 cm^{-1}; δ($CDCl_3$) 1.40 (3 H, t, *J* 7 Hz, CH_2Me), 2.15 (3 H, s, COMe), 2.24 (1 H, t, *J* 2 Hz, 5-H), 3.10 (1 H, dd, *J* 13, 2 Hz) and 3.44 (1 H, dd, *J* 13, 2 Hz) (3-H_2), 4.41 (2 H, q, *J* 7 Hz, CH_2Me), and 7.63 (1 H, s, NH) (Found: M^+, 208.0858. Calc. for $C_{10}H_{12}N_2O_3$: *M*, 208.0848); *m/z* 179, 166, 135, and 93 (100%).

2-Amino-2-(hydroxymethyl)pent-4-ynoic Acid (*α-Propargylserine*) (**11**).—Ethyl 2-amino-2-(hydroxymethyl)pent-4-ynoate (**32**) was reduced using lithium borohydride and the intermediate was hydrolysed as described above for the synthesis of (**10**). Neutralisation with 2M-aqueous ammonia yielded a tar which, in water (5 ml), was adsorbed onto a column of Dowex 50 X8 (2 × 10 cm) (H^+ form). The column was washed with water until the eluates were neutral and the amino acid was eluted from the column using aqueous ammonia (2M; 100 ml). The evaporation residue was recrystallised from aqueous ethanol to give *2-amino-2-(hydroxymethyl)pent-4-ynoic acid* (390 mg, 17%) as a white solid, m.p. 157—160 °C (Found: C, 50.0; H, 6.2; N, 9.7. $C_6H_9NO_3$ requires C, 50.3; H, 6.3; N, 9.8%); $\nu_{max.}$ 1 590, 1 620, and 2 500—3 400 cm^{-1}; δ(D_2O) 2.57 (1 H, dd, *J* 1.9, 2.4 Hz, 5-H), 2.75 (1 H, dd, *J* 17.6, 1.9 Hz) and 2.82 (1 H, dd, *J* 17.6, 2.4 Hz) (3-H_2), and 3.80 (1 H, d, *J* 11.2 Hz) and 3.95 (1 H, d, *J* 11.2 Hz) (CH_2OH); *m/z* 112 ($M^+ - CH_2OH$, 100%).

Assay for Activity of SHMT.—Cells were washed once with 0.9% aqueous sodium chloride and disrupted in the minium volume of 100mM Tris-HCl buffer at pH 7.1 using a sonicator probe at 10 KHz for 3 × 10 s. The suspension was then centrifuged at 3 000 × *g* for 1 h at 4 °C. Each assay contained the test compound, L-[3-^{14}C]serine (0.1 μmol), pyridoxal phosphate (0.1 μmol), tetrahydrofolic acid (0.8 μmol), 2-mercaptoethanol (2 μmol), and potassium phosphate buffer (pH 7.4; 30 μmol), and the supernatant from the disrupted cell preparation in a total volume of 0.2 ml. Reactions were initiated by the addition of the supernatant and were terminated by addition of sodium acetate buffer (1M; pH 4.5; 0.3 ml), aqueous formaldehyde (0.1M; 0.2 ml) and 5,5-dimethylcyclohexane-1,3-dione (0.4M in 50% aqueous ethanol; 0.3 ml). The mixture was heated for 5 min then cooled to 0 °C. Scintillation grade toluene (5 ml) was added and the bis(4,4-dimethyl-2,6-dioxocyclohexyl)-[^{14}C]methane was extracted into the toluene by vigorous shaking for 1 min. Following centrifugation, the radioactivity was determined by mixing a sample of the upper layer (3 ml) with Beckman E.P. scintillation fluid (10 ml) and counting by the liquid scintillation method on a Beckman LS-230 counter.

Growth Inhibition Assays.—The growth inhibitory effects of the compounds were determined against GM0621 and K562 cells, plated out into duplicate 1-ml wells of a 24-well plastics plate at a density of 5 × 10^4 cells ml^{-1}. R.P.M.I. 1 640 medium supplemented with 10% foetal calf serum was used as the culture medium with solutions of the compounds in the culture medium being added as appropriate. The cells were maintained under an atmosphere of 10% CO_2 in air at 37 °C. The cells were enumerated using a Coulter-counter during a 5 day incubation period and the percentage inhibition of growth was determined over the linear part of the growth curve.

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THE METABOLISM OF TRIAZENE ANTITUMOR DRUGS

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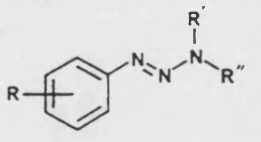
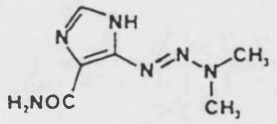
1. INTRODUCTION

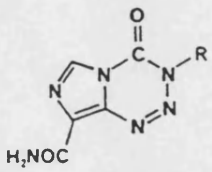
1-Aryl-3,3-dialkyltriazenes (I, Fig. 1) are carcinogens (Preussman *et al.*, 1969; Druckrey, 1973) and the dimethyl analogs (I; R′, R″ = CH_3) show potent antineoplastic activity against rodent tumors (Clarke *et al.*, 1955; Burchenal *et al.*, 1956). Use of these potently biologically active compounds has also been claimed for such diverse applications as herbicides (Mazza *et al.*, 1974), reagents for the determination of heavy metals (Popescu and Danet, 1983) and agents to render grain unpalatable to pests (Adams and Wright, 1964). Structure-antitumor activity studies on 1-aryl-3,3-dialkyltriazenes have shown that at least one *N*-methyl group is necessary for activity and that substitution in the phenyl ring has little effect on activity against the TLX5 lymphoma in mice (Audette *et al.*, 1973; Connors *et al.*, 1976). Various attempts have been made to attach the 3,3-dimethyltriazen-1-ylphenyl 'warhead' to 'delivery' moieties such as 2,4-diaminopyrimidines (Bliss *et al.*, 1987) and peptides (Ionescu *et al.*, 1981). During the last 20 yr, the role of metabolism in the mechanisms of the carcinogenicity and cytotoxicity of aryldialkyltriazenes has been studied extensively. However, many important aspects of these mechanisms remain enigmatic. One heteroaryldimethyltriazene, 5-(3,3-dimethyltriazen-1-yl)-imidazole-4-carboxamide (dacarbazine, DTIC; II, Fig. 1), is of moderate clinical usefulness in the treatment of disseminated melanoma, cancer of the colon and Hodgkin's disease which is resistant to therapy with MOPP (a combination of mechloroethamine, vincristine, procarabazine and prednisone) (Spassova and Golovinsky, 1985). Curiously, most studies of the metabolism and mechanism of the antineoplastic action of dimethyltriazenes have been carried out using substituted phenyldimethyltriazenes rather than dacarbazine itself.



I

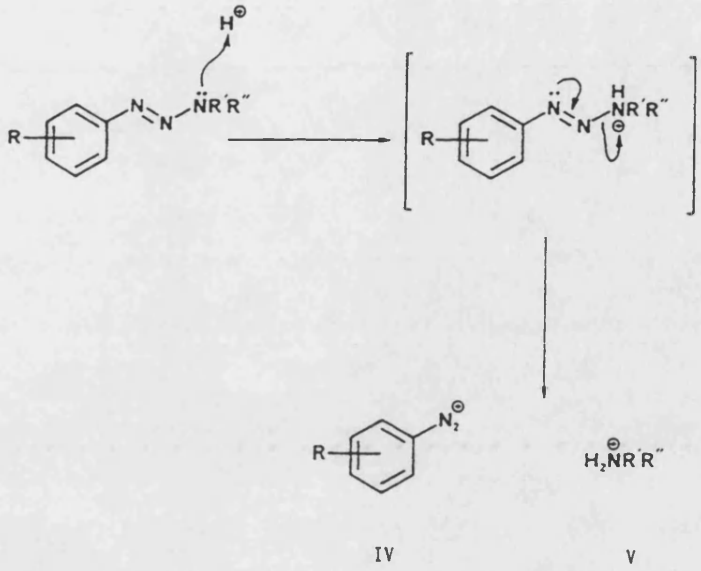


II



III

FIG. 1. Structures of triazenes and related therapeutic agents.



IV V

FIG. 2. Mechanism of acid-catalyzed chemical degradation of 1-aryl-3,3-dialkyltriazenes.

Very recently, a number of cyclic arylmonoalkyltriazenes, the 3-alkyl-8-carbamoyl-imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-ones (III, Fig. 1), have been designed as potential therapeutic alternatives to dacarbazine (Stevens *et al.*, 1984). They exhibit potent antineoplastic properties (Hickman *et al.*, 1985; Fodstad *et al.*, 1985) and two such compounds, mitozolomide [8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-one; III, R = CH_2CH_2Cl] and temozolomide [8-carbamoyl-3-methylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-one; III, R = CH_3] are currently under clinical evaluation.

The chemical properties and therapeutic aspects of 1-aryl-3,3-dialkyltriazenes have recently been reviewed extensively (Kolar, 1984; Spassova and Golovinsky, 1985). Hence, this review is principally confined to aspects of their metabolism.

In aqueous solution at physiological or mildly basic pH, 1-aryl-3,3-dialkyltriazenes are moderately stable. In contrast, acidic environments cause rapid reversal of the usual synthetic reaction (Gescher *et al.*, 1981) giving the arenediazonium salt (IV, Fig. 2) and the protonated secondary amine (V). This degradation yields potently electrophilic species and therefore this pathway could be considered to be the source of biologically reactive entities. However, no firm evidence has been put forward to support the contention that this reaction occurs at a significant rate under physiological condition.

The oxidative metabolism of 1-aryl-3,3-dialkyltriazenes by hepatic microsomes *in vitro* was first reported in 1969 (Preussman and von Hodenberg, 1969) and this study and subsequent investigations (e.g. Kolar and Schlesiger, 1976a; Kolar and Wildschütte, 1982) have left little doubt that oxidative *N*-dealkylation is a metabolic pathway pivotal for the biological properties of these compounds.

2. OXIDATIVE METABOLISM OF 3,3-DIALKYL-1-ARYLTRIAZENES IN THE ALKYL GROUPS

In the original study (Preussman and von Hodenberg, 1969) 3,3-dimethyl-1-phenyltriazene (VI, Fig. 3) was incubated with rat liver microsomes and NADPH. Formaldehyde was reported to be a metabolite and the authors suggested that the other product of this oxidation was 3-methyl-1-phenyltriazene (VII, Fig. 3). Monoalkyltriazenes such as this are powerful directly acting alkylating agents (VIII, Fig. 4) and are known to be carcinogens (Vaughan and Stevens, 1978). They react spontaneously with nucleophiles giving the corresponding arylamine (IX), molecular nitrogen and the alkylated

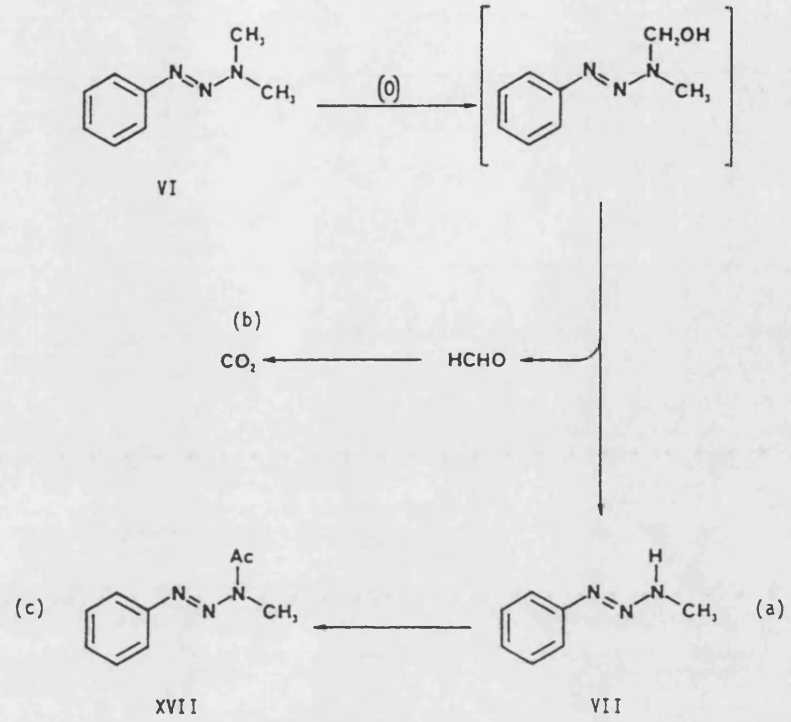


FIG. 3. Metabolism of 3,3-dimethyl-1-phenyltriazene by rodent hepatic microsomes *in vitro*. (a) Preussmann and von Hodenberg, 1969. (b) Kleihues *et al.*, 1976. (c) Pool, 1979b.

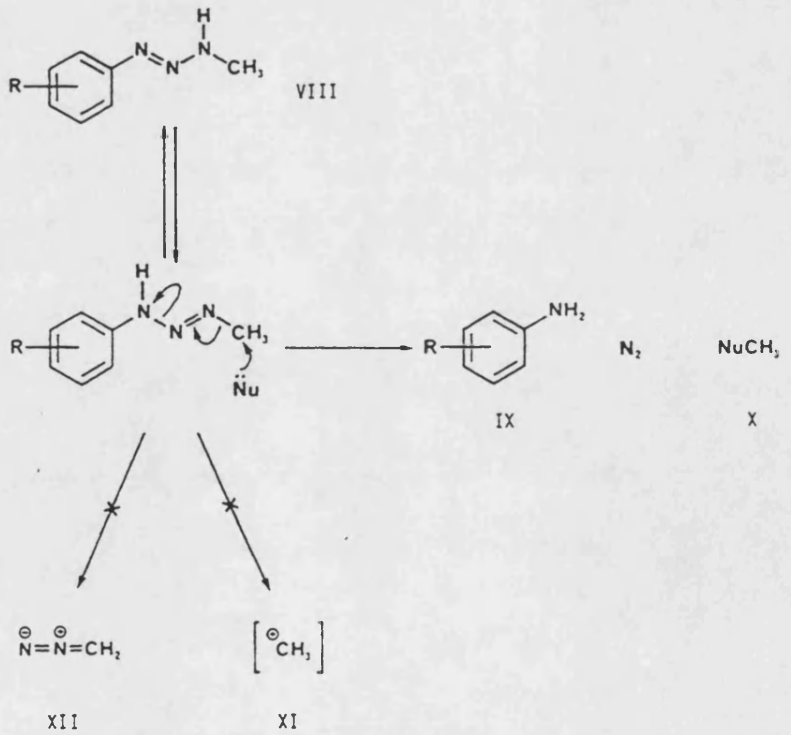


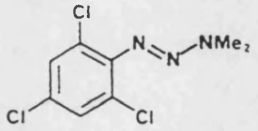
FIG. 4. Mechanism of methylation of nucleophiles by 1-aryl-3-methyltriazenes. Nu = H_2O, nucleophilic sites on DNA, proteins etc.

nucleophile (X). It can be assumed that it is this reaction mechanism which is operative in biological alkylations involving monoalkyltriazenes rather than a reaction which has been claimed to invoke the 'methyl carbonium ion' (XI), as the latter is rather unlikely to occur in biological media. Also, deuterium labeling studies have shown that diazoalkanes (XII) are unlikely to be involved as intermediates (Lown and Singh, 1981) in these alkylations. There is good evidence that the reaction between monomethyltriazenes and bionucleophiles such as nucleic acids can occur *in vivo* (Preussmann and von Hodenberg, 1969; Kleihues *et al.*, 1979; Margison *et al.*, 1979). Therefore the oxidative metabolic pathway (Fig. 3) has been postulated to be responsible for the generation of the ultimate cytotoxin which is responsible for the carcinogenicity and, possibly, the antitumor activity of aryldialkyltriazenes.

Most of the studies of the metabolism of aryldialkyltriazenes which have been conducted in the wake of the original report by Preussmann and von Hodenberg (1969) used the colorimetric determination of the aldehyde generated by hydroxylation of the *N*-alkyl group mediated by cytochrome P450 enzymes and subsequent hydrolysis/elimination. In that study, some 20 to 80% of the alkyl groups (based on the assumption that complete removal of one of the two alkyl groups equals 100%) were oxidized to aldehydes, depending upon the chemical nature of the alkyl moieties. In a later study, incubation of four (haloaryl)-dimethyltriazenes and the unsubstituted 3,3-dimethyl-1-phenyltriazene with microsomes led to the loss of between 35 and 122% of the methyl groups as formaldehyde (Poole 1979a). 3,3-Dimethyl-1-phenyltriazene yielded the lowest and 2,4,6-trichlorophenyl-3,3-dimethyltriazene (XIII, Fig. 5) gave the highest extent of oxidation at N-CH_3. These results imply some partial oxidation of the second methyl substituent. The ultimate fate of some 40% of the radioactivity administered as 3,3-di[^{14}C]methyl-1-phenyltriazene to rats was reported to be exhalation as $^{14}CO_2$ by Kleihues *et al.* (1976) who also observed the formation of 7-[^{14}C]methylguanine and other methylated DNA and RNA base residues. A study using chemical biomimetic oxidation of related triazenes in which the two alkyl groups are part of a heteroalicyclic ring has also shown facile oxidation alpha to nitrogen, although the chemical products were devoid of antitumor activity (Stevens *et al.*, 1979).

Unambiguous and direct identification of a monoalkyltriazene as a metabolite of a 1-aryl-3,3-dimethyltriazene has been reported only once; 1-(4-acetylphenyl)-3-methyltriazene (XIV, Fig. 6), together with 4-aminoacetophenone (XV), was found as a metabolite of 1-(4-acetylphenyl)-3,3-dimethyltriazene (XVI) both in the plasma of mice *in vivo* and in 9000 × *g* fractions of mouse liver homogenate *in vitro* (Farina *et al.*, 1982). A more indirect observation of a monomethyltriazene as a metabolite of a dimethyltriazene has been made in that one of the metabolites found in incubations of rodent hepatic microsomes with 1-phenyl-3,3-dimethyltriazene was 3-acetyl-3-methyl-1-phenyltriazene (XVII, Fig. 3) (Pool, 1979b). Presumably, the formation of this *N*-acyltriazene involves the monomethyltriazene (VII) as a metabolic intermediate which is subsequently acetylated.

Some doubt has been cast on the contention that an electrophile as indiscriminate and nonselective as a monomethyltriazene could account for the antineoplastic activity of dimethyltriazenes (Gescher *et al.*, 1981). This doubt was based on experiments in which the cytotoxicity of a monomethyltriazene was tested in a bioassay against a mouse TLX5



XIII

FIG. 5. Structure of 3,3-dimethyl-1-(2,4,6-trichlorophenyl)triazene.

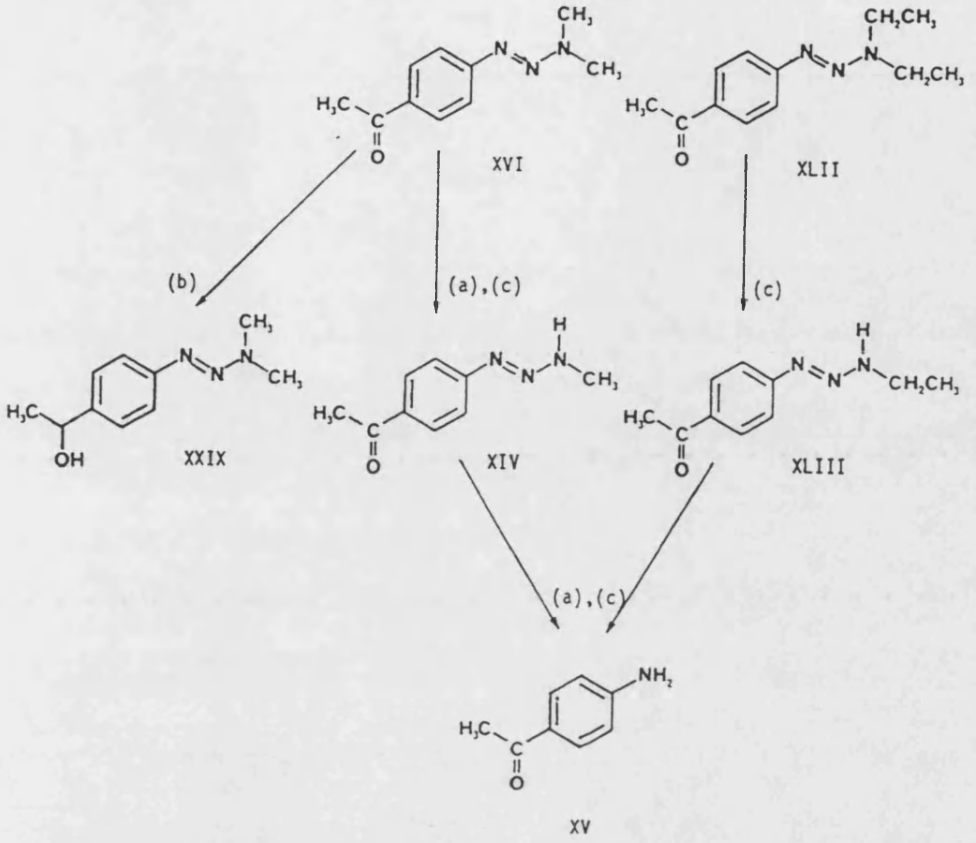


FIG. 6. Metabolism of 1-(4-acetylphenyl)triazenes. (a) Farina *et al.*, 1982. (b) Farina *et al.*, 1983. (c) Farina *et al.*, 1986.

lymphoma cell line which had acquired resistance to dimethyltriazenes *in vivo*. The monomethyltriazene was found to be nonselectively toxic to both the sensitive and the resistant lines, whereas incubation of the dimethyltriazene with a liver homogenate produced metabolites which were more toxic to the sensitive than to the resistant lymphoma cells (Gescher *et al.*, 1981). However, cytotoxic metabolites of dimethyltriazenes which are more selective than the monomethyltriazenes have yet to be isolated or characterized. The immediate metabolic precursors of 1-aryl-3-methyltriazenes, the 3-(hydroxymethyl)-3-methyltriazenes (XVIII, Fig. 7), have been synthesized (Gescher *et al.*, 1978; Juillard *et al.*, 1980) and were found to be equally as cytotoxic as their decomposition products, the monomethyltriazenes (Vaughan *et al.*, 1984; Kohlsmith *et al.*, 1984). Consequently, it has been suggested by some workers that the *N*-(hydroxymethyl) compounds may be the ultimate or proximate cytotoxic metabolites of dimethyltriazenes (Vaughan *et al.*, 1984). However, such carbinolamines have not yet been identified unambiguously as metabolites of aryldimethyltriazenes. At first sight, it is difficult to

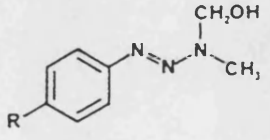


FIG. 7. Synthetic *N*-(hydroxymethyl)triazenes (Gescher *et al.*, 1978; Juillard *et al.*, 1980). $R = CO_2Me$, CO_2Et, COMe, $CONH_2$, Cl, Br, CN, NO_2.

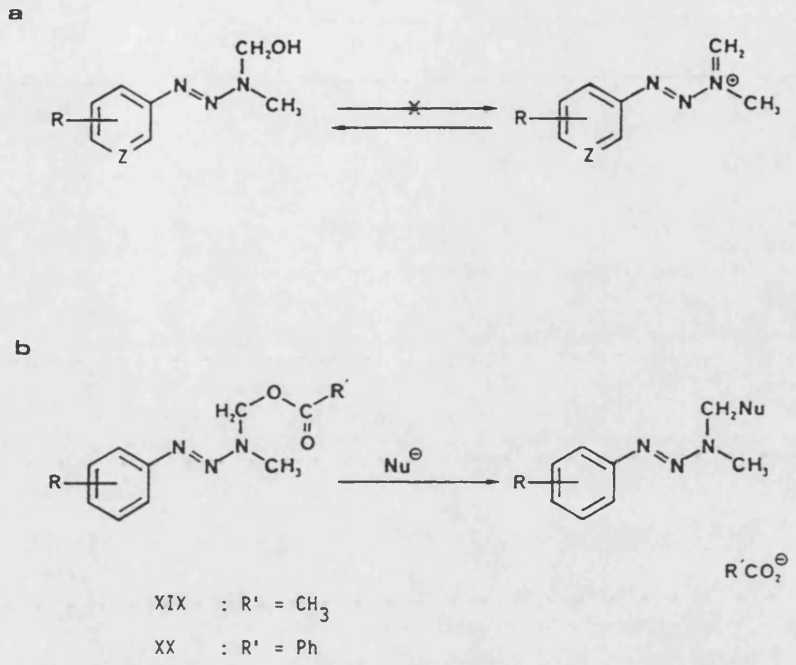
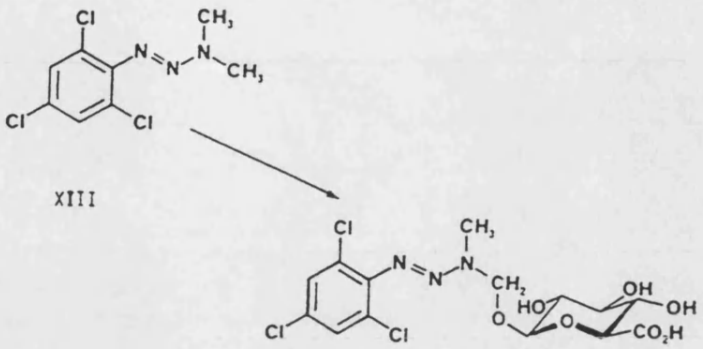


FIG. 8. (A) Lack of equilibrium between *N*-(hydroxymethyl)triazenes and the corresponding iminium ions. *Z* = CH, N. (B) Electrophilicity of a *N*-(acyloxymethyl)triazene.

envisage how such carbinolamines could be more selectively cytotoxic than their decomposition products, the monomethyltriazenes. Certain *N*-(hydroxymethyl)-amines and -amides related to *N*-(hydroxymethyl)-*N*-methyltriazenes have the ability to form, or can be in equilibrium with, electrophilic, potentially toxic imines or iminium ions, either themselves or after conjugation, and the chemical features which predispose *N*-(hydroxymethyl) compounds to undergo such reactions under physiological conditions have recently been reviewed (Overton *et al.*, 1985). Such an elimination reaction (Fig. 8A) could, on paper, be a mechanism by which hydroxymethyltriazenes exert toxicity in a manner different from that which renders monomethyltriazenes cytotoxic (Hemens *et al.*, 1984; Solway *et al.*, 1983). However, a chemical and kinetic study of the reactions of 3-(hydroxymethyl)-3-methyl-1-(pyridin-3-yl)triazene with amine bases to give 3-methyl-1-(pyridin-3-yl)triazene as the sole product (Cheng *et al.*, 1985) and the failure of the treatment of 3-(hydroxymethyl)-3-methyltriazenes with sodium cyanoborohydride, an iminium trapping agent, to yield 3,3-dimethyltriazenes (Threadgill, unpublished results) suggest that this is not an electrophilic reaction pathway open to *N*-(hydroxymethyl)-triazenes under physiological conditions. Nevertheless, it has been shown that such a reactive moiety is formed during chemical reactions of acetate and benzoate esters of *N*-(hydroxymethyl)triazenes (XIX and XX respectively, Fig. 8B); presumably the enhanced leaving-group ability of the carboxylate leads to this increased electrophilicity (Hemens *et al.*, 1984). However, such acylation of a *N*-(hydroxymethyl)triazene has yet to be shown in biochemical experiments. The rather unstable nature of *N*-(hydroxymethyl)-*N*-methyltriazenes (Vaughan *et al.*, 1984) has also been claimed to mitigate against their role as the ultimate antineoplastic species derived from dimethyltriazenes. Nevertheless, the possibility that they are important as precursors of monomethyltriazenes with a transport function cannot be excluded, particularly if they are stabilized as the corresponding glucuronide acetal conjugate. It is pertinent in this context to note that Kolar and Carubelli (1979) identified 1-([3-methyl-1-(2,4,6-trichlorophenyl)-triazen-1-yl]methyl)-β-D-glucuronic acid (XXI, Fig. 9) as a metabolite in the urine of mice which had received 3,3-dimethyl-1-(2,4,6-trichlorophenyl)triazene and the authors



XIII

XXI

FIG. 9. Metabolic oxidation/conjugation of 3,3-dimethyl-1-(2,4,6-trichlorophenyl)triazene in the mouse.

suggested that this species may, indeed, carry the monomethyltriazene to the target tissue. Conjugation of triazenes to glutathione and to sulfate has yet to be reported.

Monomethyltriazenes and 3-(hydroxymethyl)-3-methyltriazenes generated as metabolites of dimethyltriazenes could be considered as substrates for further oxidative metabolism. *N*-(Hydroxymethyl)benzamides are subject to further metabolic oxidation at the carbinolamide methylene group yielding the corresponding *N*-formylbenzamide which is excreted by the mouse in the urine (Ross *et al.*, 1983). *N*-(Hydroxymethyl)triazenes, however, have not been reported to undergo this oxidation to afford the corresponding *N*-formyltriazenes which are highly reactive entities (Ignasiak *et al.*, 1975; Threadgill and Gledhill, 1986). Evidence for a second biological oxidation has, nevertheless, been obtained in experiments in which the fate of 1-(4-acetylphenyl)-3-methyltriazene (XIV, Fig. 6) in suspensions of murine hepatocytes was investigated (Farina *et al.*, 1983). The monomethyltriazene was consumed in this medium at a rate significantly more rapid than in suspensions of nonviable cells. The chemical nature of the products of this metabolic reaction and their toxic properties are completely unknown. Oxidation at the nitrogen atoms of the triazene group of an arylmonoalkyltriazene has not been reported as a metabolic reaction, although *N*-hydroxy-*N*-methyltriazenes are relatively stable chemical entities (Freter *et al.*, 1973).

3. METABOLISM REMOTE FROM THE TRIAZENE MOIETY

It is, of course, not surprising that 1-aryl-3,3-dialkyltriazenes undergo metabolism also at sites remote from the triazene moiety, i.e. in the aryl part of the molecule or involving transformation of functional groups attached thereto. Kolar and Schlesiger (1976b) reported indirect evidence for such metabolites in that the major urinary metabolite derived from 3,3-dimethyl-1-phenyltriazene in rats was found to be 4-hydroxyaniline (XXII) with smaller amounts of the 2-hydroxy and 3-hydroxy isomers (XXIII and XXIV respectively, Fig. 10). These authors also characterized ring-hydroxylated triazenes as metabolites of this substrate, but only after chemical coupling with 1-(ethylamino)-naphthalene to give the azo dyes 1-(ethylamino)-4-(4-hydroxybenzeneazo)naphthalene (XXV) and 1-(ethylamino)-4-(2-hydroxybenzeneazo)naphthalene (XXVI). This derivatization method involves the loss of nitrogen-3 of the triazene (with its attached groups) and thus the urinary triazenes XXVII and XXVIII may have been 3,3-dimethyl, 3-methyl or even 3-(hydroxymethyl)-3-methyl compounds. A more direct example of metabolism remote from the triazene moiety with confirmed retention of the *N*-3 substituents is that the ketone carbonyl group of 1-(4-acetylphenyl)-3,3-dimethyltriazene was reduced by preparations of murine hepatic tissue *in vitro* to give 1-[4-(1-hydroxyethyl)phenyl]-

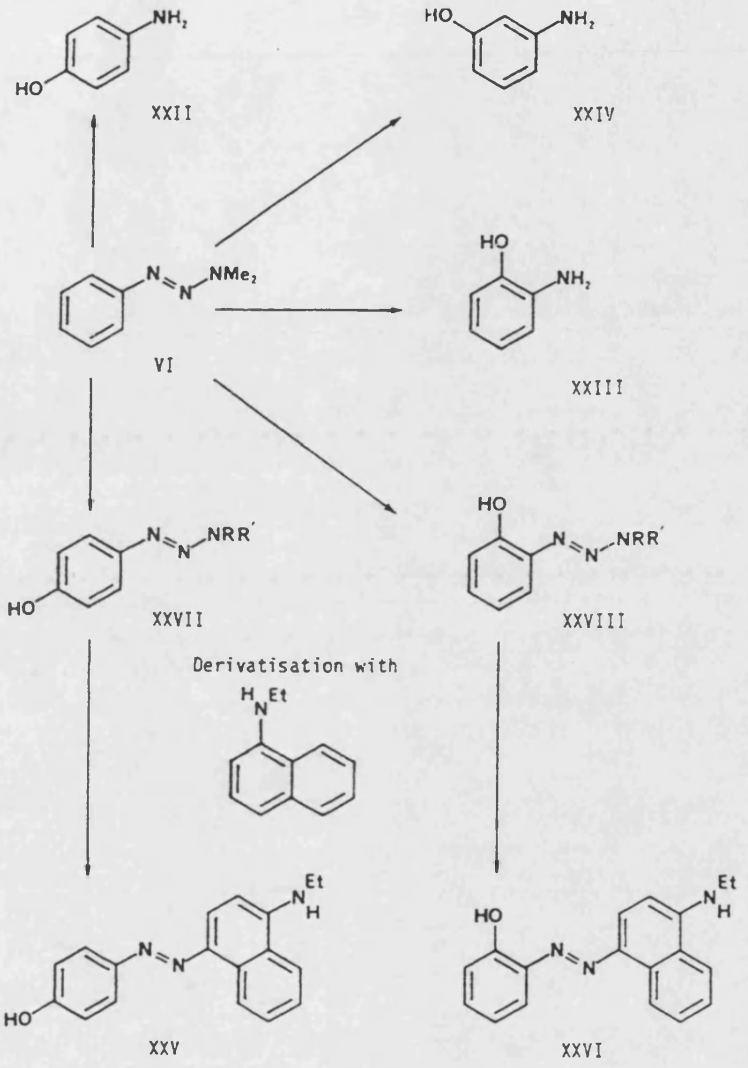


FIG. 10. Aryl-hydroxylated metabolites of 3,3-dimethyl-1-phenyltriazene in the mouse. $R = CH_3$; $R' = H, CH_2OH, CH_3$.

3,3-dimethyltriazene (XXIX, Fig. 6). Interestingly, this material retained an antitumor activity similar to that of the parent drug (Farina *et al.*, 1983).

4. METABOLIC FATE OF 1-(IMIDAZOL-5-YL)-3-ALKYLTRIAZENES

The major urinary metabolite of dacarbazine (II) in man is 5-aminoimidazole-4-carboxamide (AIC: XXX, Fig. 11) (Householder and Loo, 1969; Skibba *et al.*, 1970a,b; Breithaupt *et al.*, 1982). AIC has also been identified as a product of metabolism of dacarbazine *in vitro* in incubations with mouse liver microsomes (Hill, 1975) and with human and animal tumor tissue (Gerulath and Loo, 1972; Mizuno and Humphrey, 1972). From the mechanistic point of view, it is difficult to envisage how AIC could be formed by a metabolic pathway other than one implicating the intermediate formation of 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MTIC: XXXI), followed by the tautomerism noted by, amongst others, Hooper and Vaughan (1981) and subsequent hydrolysis. Metabolism has been reported many times to be required for activity of dacarbazine (Skibba *et al.*, 1969; Skibba and Bryan, 1971; Preussmann and von Hodenberg, 1969). The

metabolic precursor of MTIC, 5-[3-(hydroxymethyl)-3-methyltriazen-1-yl]imidazole-4-carboxamide (HMTIC, XXXII, Fig. 11) has been characterized as a urinary metabolite of dacarbazine in rats (Kolar *et al.*, 1980). Surprisingly, this compound was reported to be more stable than MTIC in polar solvents. This observation led to the suggestion that HMTIC may act as a transport form of MTIC which is the postulated ultimate antineoplastic species derived from dacarbazine. After the manner of 3-methyl-1-phenyltriazene, MTIC decomposed spontaneously in aqueous media to form AIC (XXX) and was capable of alkylating biological nucleophiles such as DNA (Mizuno and Decker, 1976).

The pharmacokinetic studies published so far on dacarbazine (Householder and Loo, 1971; Loo *et al.*, 1968; Skibba *et al.*, 1969; Breithaupt *et al.*, 1982) have not provided information on plasma or tissue concentrations of metabolically generated HMTIC or MTIC. Therefore, the pharmacokinetic data available on dacarbazine is of limited value in helping to relate the efficacy or toxicity of this drug with concentrations of cytotoxic or biologically inactive metabolites in body fluids and tissues. The major shortcoming of most of the pharmacokinetic data reported in the literature is the analytical procedure employed, a colorometric method which determines the total amount of compounds with intact triazene linkages and therefore does not discriminate between dacarbazine, HMTIC or MTIC. The plasma elimination half-life of such species after i.v. administration of dacarbazine to patients was 35 min (Loo *et al.*, 1968). Measurement of plasma concentrations of dacarbazine using a HPLC method yielded a biphasic elimination profile with a terminal half-life of 41.4 min (Breithaupt *et al.*, 1982). In this study, the plasma decay of metabolically generated AIC was monoexponential with a half-life of 43–116 min. The urinary recovery was 46–52% for dacarbazine and 9–18% for AIC. These pharmacokinetic data are in broad agreement with those obtained earlier from studies using inferior analytical methods (Householder and Loo, 1969, 1971; Loo *et al.*, 1968, 1976; Skibba *et*

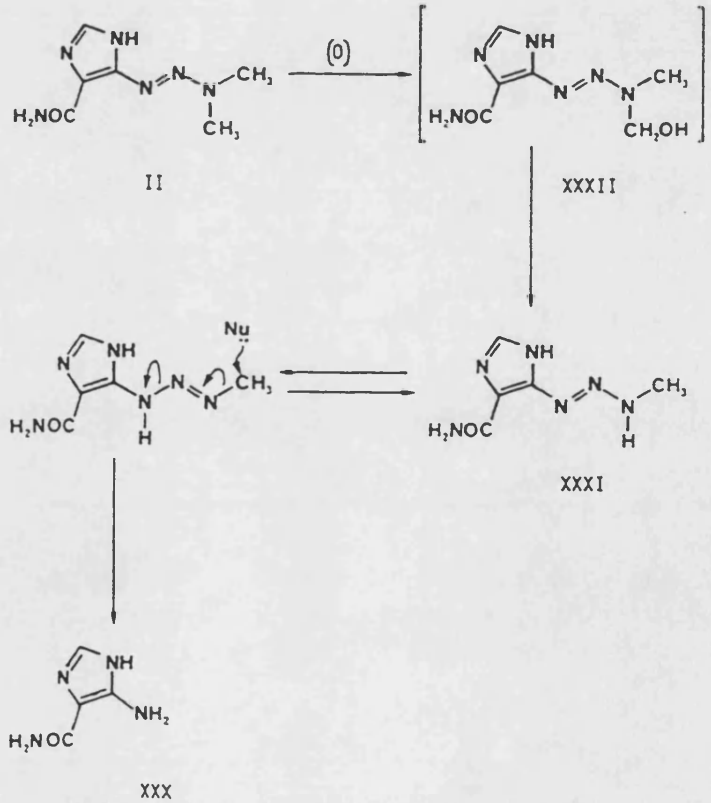
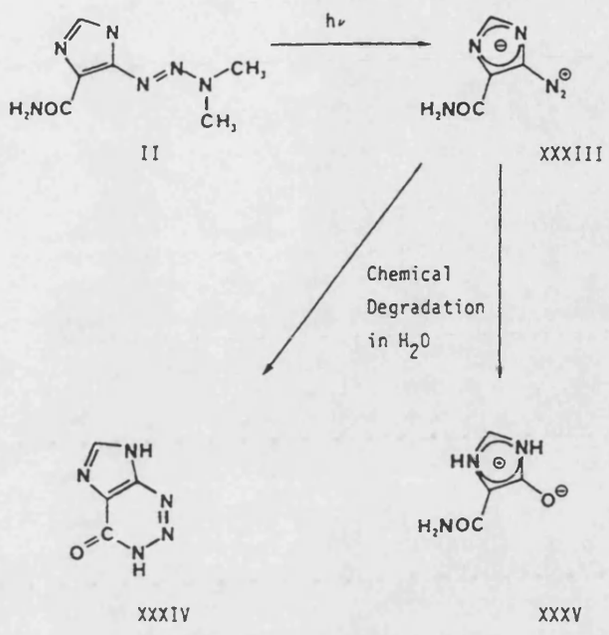


FIG. 11. Metabolism of dacarbazine. Nu = H_2O, nucleophilic sites on DNA, protein, etc.



II XXXIII XXXIV XXXV

FIG. 12. Photodecomposition and subsequent chemical degradation of dacarbazine.

al., 1969). On the basis of a preliminary pharmacokinetic study of dacarbazine and its metabolite MTIC in rodents and patients, Rutty (1983) suggested that the relative lack of activity of dacarbazine in man compared to rodents may be related to differences in the rate of hydroxylation at the *N*-methyl group of dacarbazine which is slow in man and fast in mice. It is, however, interesting to note that murine Sarcoma 180 tumor cells themselves are capable of metabolizing dacarbazine (Mizuno and Humphrey, 1972).

Dacarbazine is photolabile and reverts in light to its synthetic precursor 5-diazoimidazole-4-carboxamide (XXXIII, Fig. 12) (Horton and Stevens, 1981). This diazo compound (XXXIII) and its decomposition products, 2-azahypoxanthine (XXXIV) and 4-carbamoylimidazolium-5-olate (XXXV) have not been found as metabolites of dacarbazine *in vivo*, although the former has been implicated in the antibacterial action of the parent drug (Saunders and Schultz, 1972).

The metabolic fate of the imidazotetrazinones remains to be fully elucidated. Unlike the open-chain dacarbazine, which does not decompose under physiological conditions, the cyclic mitozolomide (XXXVI) undergoes rapid hydrolytic ring-opening to give 5-[3-(2-chloroethyl)triazen-1-yl]imidazole-4-carboxamide (MCTIC, XXXVII, Fig. 13) (Stevens *et al.*, 1984) which may well be the ultimate antineoplastic metabonate of the parent drug (Horgan and Tisdale, 1984). There is some evidence to suggest that metabolism is an important determinant of the rate of deactivation of this drug. Workman and Lee (1984) have shown that pretreatment of mice with phenobarbital reduces the activity of mitozolomide against the KHT sarcoma. In accordance with this finding, the area under the plasma concentration of mitozolomide vs time curve, determined in mice which had been pretreated with phenobarbital, was also significantly smaller than that in control animals (Brindley *et al.*, 1986). Furthermore, microsomes fortified with NADPH appeared to detoxify mitozolomide when they were added to incubation mixtures of the drug with murine TLX5 lymphoma cells (Horspool, Gescher and Stevens, unpublished).

The highly reactive and unstable mono-(2-chloroethyl)triazene MCTIC (XXXVII) which is thought to be the metabonate responsible for the antineoplastic action of mitozolomide, has a $t_{1/2}$ of only 5.5 min at pH 7.5 (Horgan, 1985). Interestingly, this is the same compound as would be speculated to be formed by metabolic oxidative dealkylation of 5-[3,3-bis(2-chloroethyl)triazen-1-yl]imidazole-4-carboxamide (XXXVIII) a dialkyl-

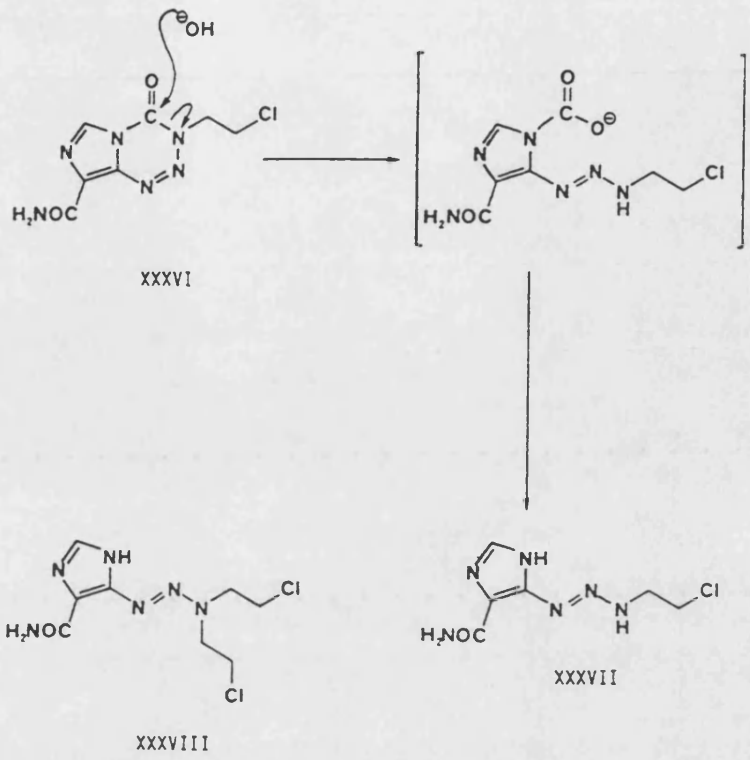


FIG. 13. Hydrolytic ring-opening of mitozolomide. Structure of 5-[3,3-bis(2-chloroethyl)-triazen-1-yl]imidazole-4-carboxamide.

triazene with activity against tumors in the brain (Shealy *et al.*, 1968; Levin *et al.*, 1975). The instability and, hence, great reactivity of 3-(2-chloroethyl)triazenes (XXXIX, Fig. 14) is thought to be due to their facile cyclization to 1,2,3-triazolines (XL) or 1,2,3-triazolinium species (XLI), (Abraham *et al.*, 1969; Lown and Singh, 1981).

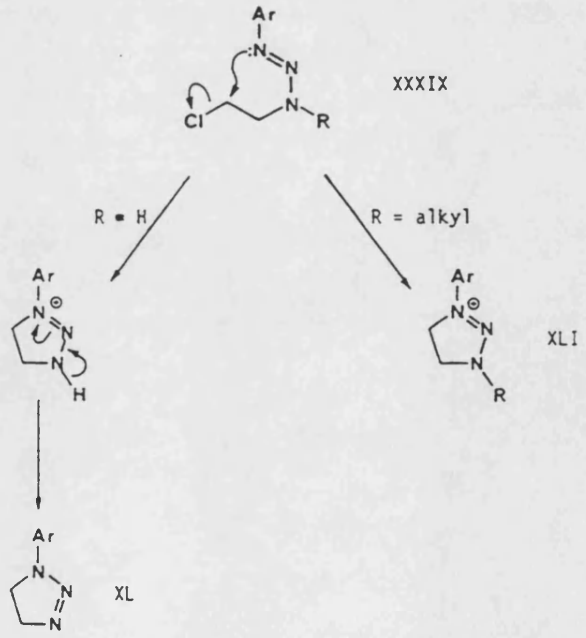


FIG. 14. Cyclization of *N*-(2-chloroethyl)triazenes.

5. THE ROLE OF METABOLISM IN THE MECHANISM OF ANTITUMOR ACTIVITY OF TRIAZENES

Many crucial mechanistic details which link the metabolism of dimethyltriazenes with their antineoplastic activity remain obscure. Answers to the following three questions appear pivotal in the elucidation of this link and require further work:

(i) How can the aggressively reactive monomethyltriazene generated by metabolism of a dimethyltriazene cause selective cytotoxicity?
(ii) Is the metabolic route which leads to the antineoplastic species identical with that which generates the carcinogenic metabolite?
(iii) Are differences in the metabolism between dimethyltriazenes and dialkyltriazines with alkyl groups other than methyl responsible for the marked difference in antineoplastic activity observed in some murine tumors (Connors *et al.*, 1976; Gescher *et al.*, 1981)?

Even though the evidence for the involvement of metabolism in the mechanism of antitumor activity of dimethyltriazenes is overwhelming, the possibility that, under certain circumstances, some dimethyltriazenes are themselves cytotoxic without metabolic activation cannot be dismissed. Dacarbazine has been used in local therapy in patients with advanced malignant melanoma and soft tissue sarcoma of the extremities and tumor regression has been observed (Aigner *et al.*, 1983). Furthermore, 1-(4-carboxyphenyl)-3,3-dimethyltriazene (I; R = 4-COOH, Fig. 1) did not undergo marked oxidative demethylation *in vitro* (Sava *et al.*, 1982), yet it possessed antimetastatic properties in mice bearing Lewis lung carcinoma (Giraldi *et al.*, 1981). This drug also increased the survival of mice bearing the TLX5 lymphoma or the P388 leukaemia without exerting marked cytotoxicity (Sava *et al.*, 1982).

The third question posed above has been addressed in a recent comparison of the antitumor activities and toxicities of 1-(4-acetylphenyl)-3,3-dimethyltriazene (XVI, Fig. 6) and 1-(4-acetylphenyl)-3,3-diethyltriazene (XLII, Fig. 6) (Farina *et al.*, 1986). The diethyltriazene was inactive against three murine tumors which were highly sensitive to the dimethyltriazene. However, the diethyltriazene was much more toxic to the host than was the dimethyltriazene. Both drugs were rapidly metabolized *in vivo* and *in vitro* and the monoalkyltriazenes (XIV and XLIII respectively) and 4-aminoacetophenone (XV) were identified as metabolites of these compounds. However, the extent to which the drugs were *N*-dealkylated differed markedly. The amount of monoethyltriazene generated was only a third of the amount of the monomethyl derivative found (Farina *et al.*, 1986). This result suggests that the difference in activities between different dialkyltriazenes may indeed by due to differences in metabolism.

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PUBLICATION 28

Cyclisation of a Substituted *N*-Acetylserine to a 2-Methyldihydroxazole with Dimethylaminosulphur Trifluoride

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CYCLISATION OF A SUBSTITUTED N-ACETYLSERINE TO A 2-METHYLDIHYDROOXAZOLE WITH DIMETHYLAMINOSULPHUR TRIFLUORIDE

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ABSTRACT: Treatment of N-acetyl-2-(benzyloxymethyl)serine methyl ester with dimethylaminosulphur trifluoride resulted unexpectedly in cyclisation to methyl 4-(benzyloxymethyl)-2-methyl-4,5-dihydro-1,3-oxazole-4-carboxylate.

As part of a programme[2] to synthesise potential enzyme-activated irreversible inhibitors of serine hydroxymethyltransferase, α-fluoromethylserine was required. Dialkylaminosulphur trifluorides carry out efficiently the conversion of alcohols to the corresponding fluoro analogues[3-5]. Amide, ester, ether and carbamate functional groups are reported[6,7] to be unaffected by these reagents. An N-acyl-O-benzylserine ester should therefore form an apposite substrate for this reagent, leading, after deprotection, to α-fluoromethylserine.

In the present work, simultaneous protection of the carboxylic acid and amino functions of O-benzylserine (1) was achieved by

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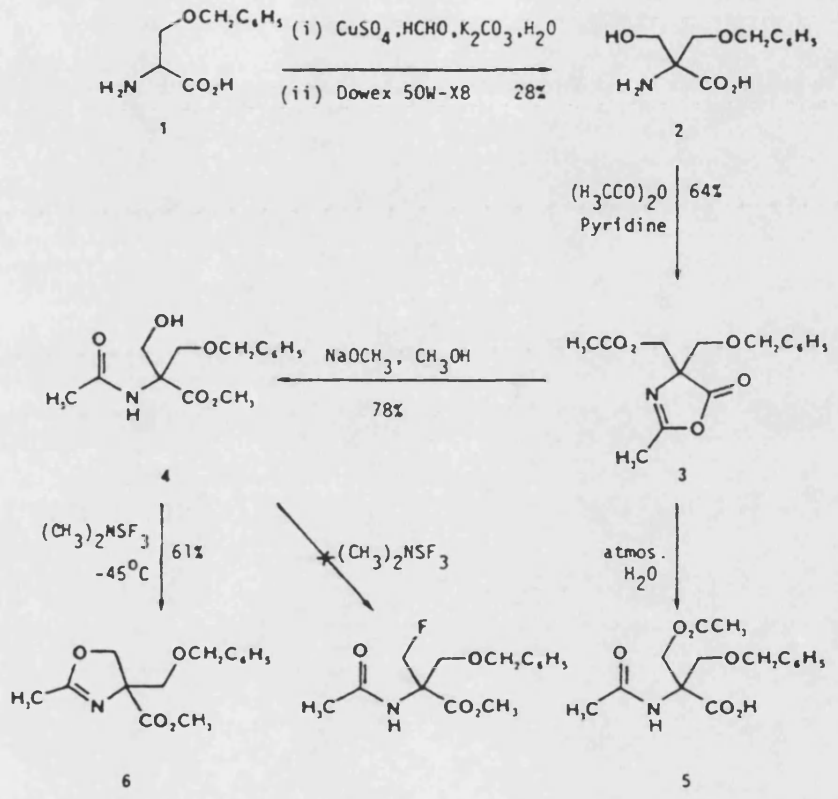
complexation with Cu^{2+}, permitting hydroxymethylation at the α-carbon by formaldehyde in aqueous basic solution[8]. After demetallation using a cation exchange resin, an adequate yield of α-(benzyloxymethyl)serine (2) was obtained as shown in the Scheme. The required substrate (4) for the dimethylaminosulphur trifluoride reaction, with protected amino and carboxylic acid groups but with a free primary hydroxyl, was easily set up in two steps. Treatment of (2) with acetic anhydride in pyridine at ambient temperature gave, as expected, acetylation at both nitrogen and hydroxyl oxygen. Raising the reaction temperature to 100^0C caused cyclisation to occur, giving the oxazol-5-one (3). Treatment with methanolic sodium methoxide removed the unwanted acetate ester by transesterification with simultaneous attack of methoxide at C-5 of the oxazolone to give (4). Compound (3) was found to be very labile to nucleophilic ring-opening, in common with related oxazol-5-ones[9], and, for elemental analysis, was characterised as the product of atmospheric hydrolysis (5). Treatment of (4) with dimethylaminosulphur trifluoride at -45^0C gave the dihydrooxazole (6) as the product of cyclodehydration, rather than the expected substitutive fluorination. Clearly, the intramolecular carbonyl oxygen is a better competing nucleophile than the exogenous fluoride ion in displacing the activated hydroxy leaving group. Interestingly, one previous report[10] concerns the dehydration/elimination of an ester of N-(benzyloxycarbonyl)threonine, another β-hydroxyamino acid, to give the 2,3-dehydro amino acid derivative, without cyclodehydration.



SCHEME

This observation should, therefore, be added to the small collection of circumstances[11,12] in which dialkylsulphur trifluorides cannot be used for conversion of OH to F by simple substitution.

EXPERIMENTAL

Melting points are uncorrected. NMR spectra were recorded using a Bruker AC300 spectrometer. Mass spectra were obtained using a VG ZAB-E instrument and infra red spectra were acquired using a Perkin-Elmer 1310 spectrometer. All compounds were racemic.

2-Amino-2-(benzyloxymethyl)-3-hydroxypropanoic acid (2). 1M Aqueous copper (II) sulphate (2.5 ml) was added to O-benzylserine (1: 5.0 g. 25 mmol) in 0.2 M aqueous K_2CO_3 (500 ml), followed by aqueous formaldehyde (38%, 15.1 ml). The mixture was boiled for 25 min then reduced to 100 ml by evaporation. The cations in the residue were adsorbed onto a column of Dowex 50-W X8 (mesh size 200-400, 2 x 10 cm; H^+ form). The resin was washed with water until neutral and the amino-acid was eluted with 2M aqueous NH_3 (100 ml). The eluates were evaporated to 5 ml and cooled to 0^0C. The precipitate was recrystallised from ethanol to give the amino-acid (2: 1.16 g. 28%), white needles MPt 164-7^0C: (Found: C, 58.7; H, 6.7; N, 6.2. $C_{11}H_{15}NO_4$ requires C, 58.7; H, 6.7; N, 6.2%): ν_{max} (Nujol) 1640, 1590, 1460 cm^{-1}: δ (CF_3CO_2D) 3.95 (1H, d, J 12 Hz) and 4.10 (1H, d, J 12 Hz) CH_2OH, 4.30 (1H, d, J 13 Hz) and 4.43 (1H, d, J 13 Hz) CH_2OCH_2Ph, 4.68 (2H, s, $PhCH_2O$), 7.25-7.60 (5H, m, ArH).

4-(Acetoxymethyl)-4-(benzyloxymethyl)-2-methyl-4,5-dihydro-1,3-oxazol-5-one (3). 2-Amino-2-(benzyloxymethyl)-3-hydroxypropanoic acid (2: 628 mg, 2.8 mmol) was heated with acetic anhydride (4.5

ml) and pyridine (3 ml) for 1h at 100°C before the mixture was evaporated to dryness at 20 torr. The residue, in chloroform, was washed rapidly with 1M hydrochloric acid and dried (anhydrous Na_2SO_4). Evaporation of the solvent gave the oxazolone (3; 519 mg. 64%), white solid MPt 121-5°C; ν_{max} (liquid film) 1825, 1740, 1690 cm^{-1}; δ ($CDCl_3$) 2.03 (3H, s, O_2CCH_3), 2.27 (3H, s, 2-CH_3), 3.70 (2H, s, CH_2OCH_2Ph), 4.13 (1H, d, J 11Hz) and 4.38 (1H, d, J 11Hz) CH_2OAc, 4.53 (2H, s, Ph_2CH O), 7.27 (5H, s, ArH). A small sample was allowed to stand in a moist atmosphere for 1 day to give 2-acetamido-3-acetoxy-2-(benzyloxymethyl)propanoic acid (5) (Found: C, 57.8; H, 6.2; N, 4.3. $C_{15}H_{20}NO_6$ requires C, 58.2; H, 6.2; N, 4.5%); m/z 310.1307 $(M+H)^+$ ($C_{15}H_{20}NO_6$ requires 310.1291).

Methyl 2-Acetamido-2-(benzyloxymethyl)-3-hydroxypropanate (4) The foregoing oxazol-5-one (3; 401 mg, 1.4 mmol) was stirred with sodium methoxide (4.35 mmol) in methanol (20 ml) for 40 min before the solvent was evaporated. The residue, in water (10ml) was acidified by addition of 4 M hydrochloric acid and extracted with dichloromethane. The extract was dried (anhydrous Na_2SO_4) and the solvent was evaporated to afford the ester (4; 301 mg, 78%), white solid MPt 118°C; ν_{max} (Nujol) 3330, 2850, 1720 cm^{-1}; δ($CDCl_3$) 2.08 (3H, s, O_2CCH_3), 3.77 (3H, s, OCH_3), 3.77 (1H, dd, J 11.6 Hz, 7.0 Hz, CHHOH), 3.81 (1H, d, J 9.3 Hz) and 3.87 (1H, d, J 9.3 Hz) CH_2OCH_2Ph, 4.01 (1H, dd, J 11.6 Hz, 5.4 Hz, CHHOH), 4.50 (1H, d, J 12.1 Hz) and 4.52 (1H, d, J 12.1 Hz) $PhCH_2O$, 4.63 (1H, dd, J 7.0 Hz, 5.4 Hz, OH), 6.59 (1H, br, NH), 7.26 (5H, s, ArH). The NMR

spin systems were confirmed using a COSY experiment. m/z 282.1358 $(M+H)^+$ ($C_{14}H_{20}NO_5$ requires 282.1341).

Methyl 4-(Benzyloxymethyl)-2-methyl-4,5-dihydro-1,3-oxazole-4-carboxylate (6). The foregoing ester (4; 144 mg, 0.5 mmol) in dichloromethane (5 ml) was added to dimethylaminosulphur trifluoride (70 mg, 0.53 mmol) in dichloromethane (3 ml) at -45^0C. The mixture was stirred at -45^0C for 30 min and allowed to warm to 20^0C before being washed (saturated aqueous $NaHCO_3$ and water) and dried (anhydrous Na_2SO_4). Preparative layer chromatography (silica gel, ethyl acetate) furnished the oxazole (6; 80 mg, 61%), oil; ν_{max} (liquid film) 1720, 1640 cm^{-1}; δ ($CDCl_3$) 2.00 (3H, s, 2-CH_3), 3.54 (1H, d, J 9.2 Hz, C<u>H</u>HOCH_2Ph), 3.77 (3H, s, OCH_3), 3.84 (1H, d, J 9.2 Hz, CH<u>H</u> OCH_2Ph), 4.31 (1H, d, J 8.9 Hz, 5-H), 4.55 (2H, s, PhCH_2O), 4.71 (1H, d, J 8.9 Hz, 5-H), 7.26 (5H, s, ArH); m/z (Chemical ionisation) 264.1239 $(M+H)^+$ ($C_{13}H_{18}NO_4$ requires 264.1236).

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PUBLICATION 29

Applications of Tandem Mass Spectrometry to the Characterization of Derivatized Glutathione Conjugates. Studies with *S*-(*N*-Methylcarbamoyl)glutathione, a Metabolite of the Antineoplastic Agent *N*-Methylformamide

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Applications of Tandem Mass Spectrometry to the Characterization of Derivatized Glutathione Conjugates. Studies with *S*-(*N*-Methylcarbamoyl)-glutathione, a Metabolite of the Antineoplastic Agent *N*-Methylformamide

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Daughter ion spectra are reported for $[M + H]^+$ ions generated by fast atom bombardment mass spectrometry of *S*-(*N*-methylcarbamoyl)glutathione (1) and a series of alkoxycarbonyl methyl ester derivatives thereof. Structurally informative, even-electron fragment ions, which serve to define the nature of both the xenobiotic and peptide components of the conjugate, are observed in the collisionally activated dissociation (CAD) spectra of 1 and its ethoxy- and benzyloxycarbonyl methyl esters. Studies with the *t*-butyloxycarbonyl (*t*BOC) methyl ester derivative, on the other hand, indicated that the *t*BOC group exerts a powerful directing influence on the CAD process, and that the major daughter ions in this case are associated with cleavage of the *t*BOC functionality itself and are of little diagnostic value. Of the derivatives examined, the benzyloxycarbonyl congener, which may be generated readily from 1 in aqueous media, is judged to be the most useful from the standpoints of ease of formation, desirable high-performance liquid chromatographic properties, and informative mass spectral fragmentation characteristics under CAD conditions.

INTRODUCTION

Conjugates of the endogenous tripeptide glutathione (γ-L-glutamyl-L-cysteinylglycine, GSH) represent an important class of metabolites since they are formed from a wide variety of biological electrophiles.[1-3] In many cases, such electrophilic intermediates, which typically are generated as short-lived metabolites of drugs or other foreign compounds, have been implicated as causative agents in various xenobiotic-induced toxicities. Consequently, the development of methods to isolate and characterize GSH adducts has become an important goal in biochemical toxicology, since knowledge of the structure of a given conjugate provides a unique insight into the nature of the reactive metabolite from which it derives.[4,5]

Mass spectrometric analyses of GSH conjugates, however, have been hampered by the high polarity, thermal instability and, in certain instances, the chemical reactivity of these compounds, which have combined to preclude study of all but the simplest examples by traditional electron impact (EI) or chemical ionization (CI) techniques.[6] On the other hand, 'soft' ionization methods, e.g. field desorption[7-12] and, more recently, fast atom bombardment (FAB) or liquid secondary ion (LSI) mass spectrometry[13-22] and liquid chromatography/thermospray mass spectrometry[23,24] have provided highly effective means of examining GSH conjugates as intact species, and have been applied successfully to the mass spectrometric analysis of numerous members of this structural class.[25] However, although abundant $[M + H]^+$ and $[M + Na]^+$ ions normally are produced under such conditions, few fragment ions are observed from which details of molecular structure may be inferred. Very recently, collisionally activated dissociation (CAD) methods, employed in conjunction with tandem mass spectrometry (MS/MS), have been used to overcome this problem, and have been applied to the characterization of selected GSH conjugates of synthetic or biological origin.[26-30]

A complementary approach to the analysis of thioether adducts, which has proven to be of considerable value in studies performed in our laboratories,[31,32] involves conversion of the conjugates (in aqueous media, e.g. bile) into the corresponding alkoxycarbonyl derivatives in order to facilitate their initial isolation from biological matrices, and to assist in their subsequent purification prior to mass spectrometric analysis. By this procedure, derivatized drug–GSH conjugates, such as that of the analgesic agent acetaminophen, were readily isolated in pure form and yielded both protonated molecular species and a limited number of daughter ions when analysed by field desorption mass spectrometry or by LSI mass spectrometry with Cs^+ ion bombardment.[33]

The objective of the present investigation was to extend the above derivatization approach to a study of the behavior of functionalized GSH conjugates upon CAD, in an attempt to extract detailed structural infor-

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mation from their FAB spectra through tandem mass spectrometric analysis of the family of daughter ions produced. As a model compound for these experiments, we chose *S*-(*N*-methylcarbamoyl)glutathione (**1**), the GSH conjugate of the experimental anti-tumor agent *N*-methylformamide (**2**) (Fig. 1).[34] In addition to providing a means of assessing the influence of alkoxycarbonyl formation on the pathways of collision-induced fragmentation of the GSH moiety, selection of this model compound afforded an opportunity to compare the CAD spectra reported recently[30] for *S*-alkyl and *S*-aryl GSH conjugates with that of the novel *S*-carbamoyl-GSH structure of **1**. Moreover, since **1** had to be generated biochemically for these studies by metabolism of **2** in isolated rat hepatocytes, it was possible in this investigation to make a realistic assessment of the practical value of the combined aqueous-phase derivatization and FAB mass spectrometry (or FAB MS/MS) approach to the analysis of GSH conjugates in biological specimens.

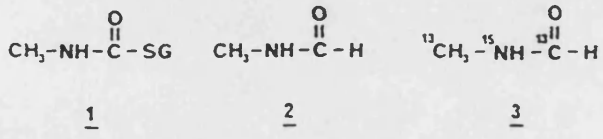
EXPERIMENTAL

Materials

N-Methylformamide (NMF) (**2**), ethylchloroformate, benzylchloroformate and reagents for synthetic and analytical work were purchased from the Aldrich Chemical Co. (Milwaukee, Wisconsin). [$^{13}C_2$,^{15}N]NMF (**3**), with an isotopic purity of 90 atom% excess $^{13}C_2$ and 95 atom% excess ^{15}N, was obtained from MSD Isotopes (St Louis, Missouri), while *N*-[^{14}C-methyl]-formamide (specific activity 1 mCi mmol^{-1}, radiochemical purity 97%) was synthesized by treatment of [^{14}C]methylamine hydrochloride (ICN, Irvine, California) with excess ethyl formate, as described previously.[35] Collagenase (grade II) was obtained from Worthington Biochemical (Freehold, New Jersey). A sample of the *t*-butyloxycarbonyl (*t*BOC) methyl ester derivative **7** was prepared by total synthesis, full details of which will be reported elsewhere.[36]

Biological experiments

Hepatocytes were isolated from male Sprague–Dawley rats (200–250 g; Charles River Laboratories, Wilmington, Massachusetts) by a collagenase perfusion technique.[37] Cell viability, determined by Trypan Blue exclusion, was typically between 85 and 90%. Incubations (2×10^6 cells ml^{-1}), with **2** or **3** as substrate (5 mM), were carried out in Krebs–Henseleit buffer (pH 7.4,



1 2 3

Figure 1. Structures of the GSH conjugate of NMF (**1**), and of unlabeled (**2**) and $^{13}C_2$,^{15}N-labeled NMF (**3**).

final volume 20 ml) under an atmosphere of O_2/CO_2 (95/5). (^{14}C)NMF (diluted for metabolic experiments, with **2** or **3**, to a specific activity of 18.4 μCi mmol^{-1}) was added to hepatocytes at the outset, and incubations were performed for 4 h at 37 °C in an orbital incubator. Metabolism was terminated by the addition of two volumes of ice-cold acetone, and the precipitated protein was removed by brief centrifugation (500 × *g*). Acetone was removed from the supernatant under reduced pressure, and the residual aqueous phase, which contained **1** (or its $^{13}C_2$,^{15}N-labeled analog), was treated as outlined previously[34] with excess ethyl- or benzylchloroformate, and extracted using a Sep-Pak cartridge (Waters Associates).[34] Samples of these derivatized extracts were taken directly for analysis by high-performance liquid chromatography (HPLC).

HPLC

Purification of alkoxycarbonyl derivatives was performed using a Beckman model 342 dual pump instrument, equipped with a reverse-phase Novapak ODS column (15 cm × 4.6 mm i.d., 4 μm; Waters Associates, Milford, Massachusetts) and fixed-wavelength (λ = 254 nm) Beckman model 160 ultraviolet (UV) detector. The mobile phase consisted of a linear 20 min gradient from 20% to 70% methanol in water with 1% acetic acid throughout. The flow rate was 1.0 ml min^{-1} and 1 min fractions were collected and evaporated to dryness. Under these conditions, the ethoxy- and benzyloxycarbonyl derivatives of **1** eluted in fractions 5–6 and 11–12, respectively. The recovery of radioactivity in the HPLC eluent was >95% of that applied to the column, based on counting of fractions on a Beckman LS-7500 liquid scintillation spectrophotometer. The purified conjugates thus obtained were treated with anhydrous methanolic HCl at room temperature for 2 h, and the resulting alkoxycarbonyl methyl esters were subjected to HPLC purification using the above system. The fractions eluting from the column between 10–11 and 15–16 min (which contained **4** and **5**, respectively) were collected, evaporated to dryness, and taken for mass spectrometric analysis.

Mass spectrometry

Mass spectrometric analyses were performed using a VG 70 SEQ hybrid tandem instrument of *EBQQ* geometry (VG Analytical Ltd, Manchester, UK), equipped with an Ion Tech fast atom gun and VG 11/250 data system. Samples (5–10 μg) were dissolved in methanol (3 μl) and added to glycerol (3 μl) containing 0.1 M HCl (1 μl) on an FAB target. Ionization was achieved following bombardment with xenon (6 keV) as the primary beam, and conventional FAB spectra were recorded, at an accelerating potential of 8 kV, via the data system.

CAD was performed in the first (rf-only) quadrupole region, using helium as the collision gas with collision energies of 38–40 eV. The pressure in the quadrupole analyser housing was maintained at 1×10^{-6} Torr, and daughter ion spectra were obtained by selection of the appropriate $[M + H]^+$ parent ion through adjustment

of the magnetic field, and scanning the quadrupole mass analyser from m/z 600 to 50 over a period of 10 s. Daughter ion spectra were recorded using an oscillographic recorder.

RESULTS AND DISCUSSION

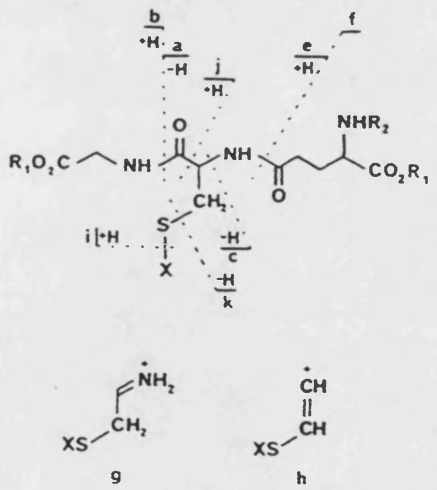
Preparation of alkoxycarbonyl derivatives

Reaction of metabolite **1** with ethyl- or benzylchloroformate in buffered aqueous solution afforded the respective ethoxy- and benzyloxycarbonyl derivatives in essentially quantitative yields. The benzyloxycarbonyl derivative of **1** was, as expected, appreciably more lipophilic than its ethoxy counterpart. This property, coupled with the introduction of a chromophore which permitted detection of the conjugate by UV absorbance, favored the benzyloxycarbonyl derivative for use in the purification of the NMF conjugate by reverse-phase HPLC techniques.

FAB mass spectrometry

Conventional FAB mass spectrometric analysis of the native conjugate **1**, and of derivatives **4**–**7**, afforded spectra with prominent $[M + H]^+$ ions, similar to those reported for *S*-alkyl and *S*-aryl GSH conjugates.[13–22,30] In some cases, structurally informative fragmentation was noted, e.g. compound **1** exhibited even-electron daughter ions resulting from cleavage of the γ-glutamyl–cysteinyl peptide bond with charge retention on the residual drug conjugate (ion *e*, Scheme 1), and from elimination of the elements of methylisocyanate (CH_3NCO) from the NMF moiety (ion *i*, Scheme 1). Modification of the conjugate by formation of alkoxycarbonyl methyl ester derivatives, on the other hand, led to preferred cleavage of the cysteinyl–glycine linkage to yield ion *a* (Scheme 1). Similar changes in fragmentation following derivative formation were observed under CAD conditions (see below). The *t*BOC methyl ester **7** was unique among the compounds examined in this study in that abundant daughter ions resulting from cleavages involving the alkoxycarbonyl group were evident in the FAB mass spectrum; these ions corresponded to the $[M + H - C_4H_8]^+$ and $[M + H - CO_2C_4H_8]^+$ species typically observed in the FAB spectra of *t*BOC-protected peptides.[38]

Despite the appearance of daughter ions in the conventional FAB spectra of **1** and its derivatives, these fragments generally were of low abundance and frequently were obscured by interfering components of the sample matrix. The spectrum reproduced in Fig. 2(a), which was obtained by FAB analysis of the benzoxycarbonyl methyl ester derivative **5** isolated from rat hepatocytes, may be taken as a representative example.



Scheme 1. Pathways of fragmentation of glutathione conjugates following collisional activation. The identities of substituents R_1, R_2 and X are given in Table 1. By convention, proton attachment or elimination reactions are depicted with respect to the neutral parent molecule, rather than the $[M + H]^+$ ion.

FAB MS/MS

When the $[M + H]^+$ ions from the native and derivatized GSH conjugates were subjected to CAD, a series of even-electron daughter ion spectra were generated whose salient features are summarized in Table 1. The proposed origins of fragments *a*–*k* are depicted in Scheme 1, which is adapted from recent work by Gaskell and co-workers[30] and employs the same notations for ion structures. Shifts in the m/z values of daughters produced either by changes in the alkoxycarbonyl substituent or by isotopic labeling of the NMF moiety served to support these proposed assignments.

In the case of the underivatized conjugate (**1**), loss of the γ-glutamyl moiety (129 u) to give ion *e* represented the major route of fragmentation, and was accompanied by further elimination of the elements of water to yield the prominent $[e - H_2O]^+$ ion at m/z 218. These cleavages have been described for many other native GSH conjugates analysed by 'soft' ionization methods, with and without CAD, and appear to be characteristic of this structural class.[10,26,29,30,33] Other significant daughter ions in the CAD spectrum of **1** resulted from expulsion of the elements of methylisocyanate (CH_3NCO), either directly from the $[M + H]^+$ (to give *i*) or from the above *e* fragment (to yield m/z 179), both of which served to confirm the composition of the xenobiotic moiety attached directly to sulfur. Interestingly, this pathway of ion decomposition is absent in the CAD spectra of both *S*-alkyl and *S*-aryl GSH conjugates,[30] and may therefore have some diagnostic importance for the 'H—Y—CO—SG' partial structure (where 'Y' is a heteroatom). Indeed, preliminary CAD studies on a derivative of *N*-ethylcarbamoylcysteine (which eliminates the elements of ethylisocyanate) and on an *N*,*N*-dimethylcarbamoylcysteine conjugate (which does not fragment by such a pathway) serve to support this contention (data not shown). Thus, daughter ions indicative of the structure of both drug and tripeptide elements of the GSH conjugate **1** were clearly evident in the CAD spectrum of its $[M + H]^+$ parent.

Conversion of **1** to its methyl ester ethoxy- (or benzyloxy)carbonyl derivative induced a pronounced change in the relative abundances of several of the ions *a*–*k*, most notably in the intensity of fragment *a*

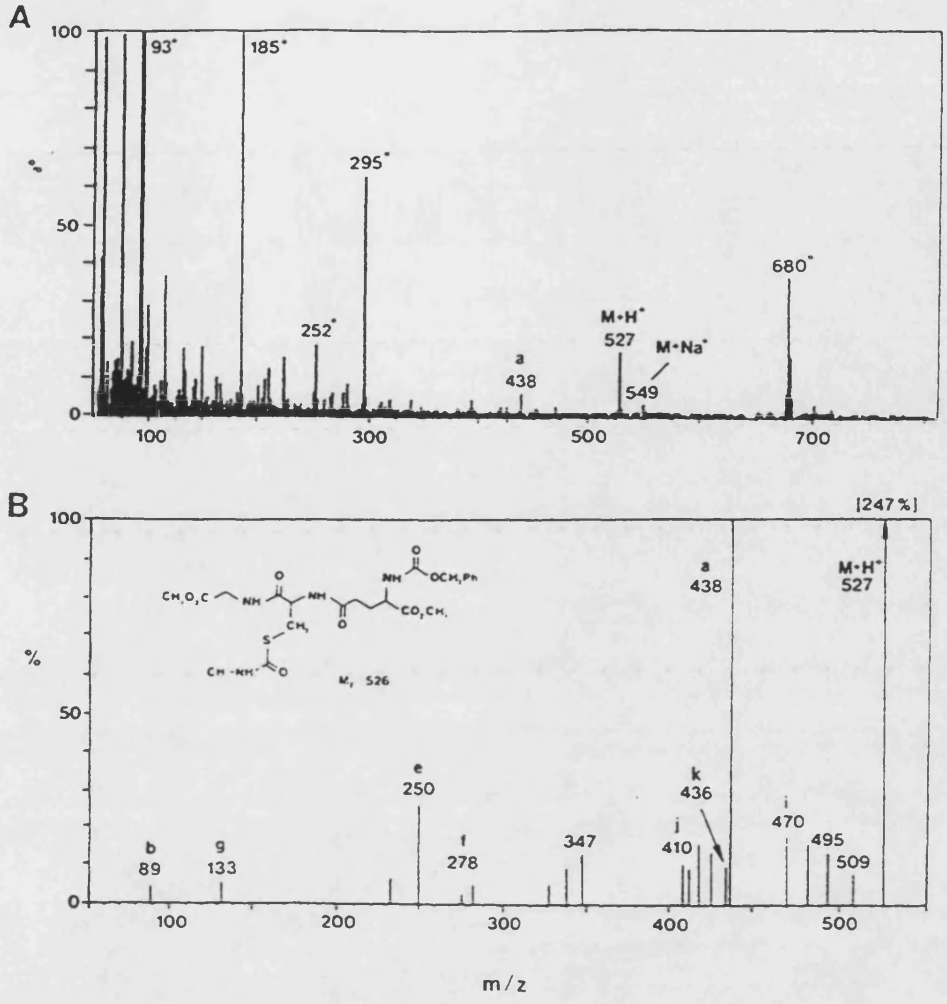


Figure 2. FAB mass spectrometric analyses of a purified sample of the benzoxycarbonyl methyl ester derivative **5**, isolated from an incubation of NMF with rat hepatocytes. The conventional FAB spectrum is shown in (a), which contains numerous extraneous ions (denoted by asterisks) from components of the matrix and from the biological isolate, while (b) depicts the daughter ion spectrum of *m/z* 527 ($[M + H]^+$), obtained from the same sample. The identities of daughter ions *a–k* are indicated in Scheme 1.

(elimination of the elements of glycine methyl ester) which dominated the CAD spectra of compounds **4–6** (Table 1). (Similar changes have been reported to accompany *N*-acetylation of model GSH conjugates, e.g. *S*-octylglutathione.[30] However, loss of the γ-glutamyl residue (to give *e*) remained an important route of fragmentation of these derivatives, as did expulsion of methylisocyanate (57 u) from the respective $[M + H]^+$ ions. In addition, losses of small neutral molecules from the $[M + H]^+$ parent proved to be a characteristic feature of the CAD spectra of compounds **4–6**, and were ascribed to reactions involving the methyl ester (−MeOH) or alkoxycarbonyl functionalities (−EtOH, $-PhCH_2OH$, $-CO_2$). The daughter ion spectra of the ethoxy- and benzyloxycarbonyl derivatives, therefore, reflected the nature of the drug residue, the GSH moiety, and the functionalized amino and carboxyl groups of the conjugate. These features are illustrated in the CAD spectrum of the benzyloxycarbonyl methyl ester **5**, which is shown in Fig. 2(b). In striking contrast to the spectra of daughter ions produced from the ethoxy- and benzyloxycarbonyl methyl esters of **1**, that recorded from the *t*BOC methyl ester **7** was dominated by two ions which resulted from fragmentation of the *t*BOC group itself, viz. *m/z* 437 ($[M + H - C_4H_8]^+$) and *m/z* 393 ($[M + H - CO_2C_4H_8]^+$). These species, which also were evident in the conventional FAB spectrum of **7** (see above), may be regarded as characteristic products of collisional activation of peptide *t*BOC derivatives[38] and convey little information on molecular structure. Only ions *a* and *e* provided any such information in this case, although their low relative abundances would limit the potential utility of this *t*BOC derivative for analytical purposes.

CONCLUSIONS

The results of these FAB mass spectrometric and FAB tandem mass spectrometric studies with the GSH conjugate of NMF indicate that adducts with the novel *S*-

Table 1. Daughter ion spectra of $[M + H]^+$ ions from *S*-(*N*-methylcarbamoyl)glutathione (1) and derivatives thereof

No.	R_1	R_2	Substituent X	m/z values and (in parentheses) relative abundances of characteristic daughter ions[a]																				Other ions
				$[M+H]^+$	$[M+H]^+$ $-H_2O$	$[M+H]^+$ $-MeOH$	$[M+H]^+$ $-CO_2$	$[M+H]^+$ $-EtOH$	$[M+H]^+$ $-PhCH_2OH$	*a*	*b*	*c*	*d*	*f*	*g*	*h*	*i*	*j*	*k*					
1	H	H	$CH_3-NH-CO-$	365 (323)	347 (10)	—	—	—	—	290 (10)	75 (19)	—	236 (100)	130 (28)	133 (45)	116 (10)	308 (16)	—	274 (3)	218[b] (58)	199[c] (13)	179[d] (22)		
4	Me	CO_2Et	$CH_3-NH-CO-$	465 (455)	447 (18)	433 (23)	—	419 (27)	—	376 (100)	89 (9)	233 (18)	250 (41)	216 (46)	133 (23)	—	408 (77)	348 (14)	374 (9)	382[b] (32)	351[c] (18)	285[d] (36)		
5	Me	CO_2CH_2Ph	$CH_3-NH-CO-$	527 (247)	509 (8)	495 (14)	483 (16)	—	419 (16)	438 (100)	89 (4)	—	260 (26)	278 (3)	133 (5)	—	470 (18)	410 (8)	436 (10)	426[b] (13)	413[c] (9)	347[d] (14)		
6	Me	CO_2CH_2Ph	$^{13}CH_3-^{15}NH-^{13}CO-$	530 (212)	512 (10)	498 (23)	486 (23)	—	422 (18)	441 (100)	89 (8)	—	263 (35)	278 (8)	136 (10)	—	470 (35)	413 (23)	436 (11)	426[b] (16)	416[c] (15)	347[d] (22)		
7	Me	CO_2tBu	$CH_3-NH-CO-$	493 (333)	475 (7)	—	449 (3)	—	—	404 (7)	—	—	250 (3)	—	—	—	—	—	—	437[b] (20)	393[c] (100)	—		

[a] Ion abundances are expressed relative to the most intense daughter ion. (See Scheme 1 for identities of ions *a*–*k*).
[b] $[a - H_2O]^+$; [c] $[a - CH_3NCO]^+$; [d] $[a - CH_3NHCOSH]^+$; [e] $[419 - CH_3NCO]^+$; [f] $[M + H - 114]^+$; [g] $[M + H - 180]^+$ ([h] $[M + H - 183]^+$ in spectrum of 6); [i] $[i - CO_2]^+$; [j] $[M + H - C_4H_8]^+$; [k] $[M + H - CO_2C_4H_8]^+$.

carbamoylglutathione structure behave in a generally similar fashion to their more widely encountered *S*-alkyl and *S*-aryl counterparts. However, fragmentation involving loss of the xenobiotic moiety with accompanying hydrogen transfer to the charged species (ion *i*) appears to be unique to this group of metabolites, where it should prove to be of diagnostic utility.

Formation of alkoxycarbonyl methyl ester derivatives of 1 had a marked directing influence on the CAD spectra of the $[M + H]^+$ ion, a feature which should prove to be of value in the design of experiments to detect specific GSH conjugates in biological samples by FAB mass spectrometric techniques. Thus, by analogy with the proposed use of constant neutral loss scanning for 129 u (formation of ion *e*) as a means of screening complex mixtures for *native* GSH adducts,[26,30] neutral loss scans for elimination of both the derivatized glycinyl and γ-glutamyl residues (formation of ions *a* and *e*, respectively) in the CAD spectra of alkoxycarbonyl methyl esters should, in principle, provide an increased degree of specificity for the detection of such conjugates.

Finally, this work has confirmed and extended the findings of others[26,29,30] who have reported on the value of tandem mass spectrometric techniques for the characterization of GSH conjugates, and further indicates that the combination of aqueous phase derivatization procedures[33] and contemporary mass spectrometric methods represents a powerful approach to the study of this toxicologically important class of xenobiotic conjugates.

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A Note on

NMR AS AN AID IN STUDYING *N*-ALKYLFORMAMIDES AND METABOLITES†

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The common industrial and laboratory solvent DMF‡ and its close analogue NMF share the ability to cause hepatotoxicity in man. They differ, however, in their biological properties towards rodents. NMF is a hepatotoxin towards rats and mice and shows significant activity against tumours of murine [1] and human [2] origin grown in mice. DMF shows neither strong therapeutic nor hepatotoxic activity in mice, whereas NEF in mice shows hepatoxicity only [1, 3]. The explanation for these comparisons and contrasts may be connected with the nature and quantity of the metabolites formed from different *N*-alkylformamides in the various species. The characterization of the urinary metabolites by conventional techniques such as MS and UV spectrometry is hampered by their lability and lack of a suitable chromophore.

NMR spectroscopy was used to overcome these problems in two ways. Firstly, ^{1}H-NMR spectra were obtained using a Bruker WH400 spectrometer at 400 MHz on 0.5 ml samples of urine from mice to which DMF, NMF or other analogues had been administered at ~7.0 mmol/Kg. No other manipulation such as derivatization was involved, but 0.05 ml D_2O was added so as to provide a lock signal for the spectrometer. Sodium 3-(trimethylsilyl)propan-1-sulphonate was also added, as an internal chemical shift standard. Suppression of the H_2O signal (δ 4.80) was achieved by selective presaturation (1.5 sec) and was followed by collection of the free induction decay after 4 dummy

†See also J.K. Nicholson et al., art. #D-1 in Vol. 16, this series.– *Ed.*

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‡*Abbreviations.*– Formamide derivs.: *N*,*N*-dimethyl, DMF; *N*-methyl, NMF; *N*-ethyl, NEF; *see over for* HMMF. *N*-acetyl-*S*-(*N*-methylcarbamoyl)cysteine, AMCC; if Et, not Me: AECC.

scans. This technique permitted satisfactory ^{1}H spectra to be recorded for the δ ranges 0–4 and 6–11 and so resonances arising from most of the protons of the various metabolites of the formamides could be resolved, with the exception of those of $\underline{NCH_2}OH$ groups which have chemical shifts in the δ range 4.3–5 [4].

By comparison of the chemical shifts of the CHO protons(δ 8.03 for Z rotamer, 7.93 for E) and NCH_3 protons (δ 3.05 Z and 2.91 E) in synthetic *N*-(hydroxymethyl)-*N*-methylformamide (HMMF) with those of signals in the urine of mice which had been treated with DMF, it was possible to show [4] that the major urinary metabolite of DMF in rodents is HMMF. This product is degraded to NMF under GC and MS conditions, leading to previous reports [5, 6] of NMF as the major metabolite of DMF; this demonstrates the value of the non-destructive nature of direct NMR analysis.

Similarly, *N*-(hydroxymethyl)acetamide was shown to be the almost exclusive urinary metabolite of *N*-methylacetamide in mice [3]. The corresponding direct ^{1}H-NMR spectra of urine of mice which had received NMF and NEF indicated the presence of the parent compound and the corresponding amine (methylamine and ethylamine respectively). Additionally [cf. observations by J.K. Nicholson et al. (p. 331 in Vol. 16)- *Ed.*], signals corresponding to the SCONHalkyl and $NCOCH_3$ protons of the corresponding mercapturic acids (AMCC and AECC) were observed in thse direct urine spectra, as shown in Fig. 1.

The two principal drawbacks encountered by us with the above straightforward direct method were insufficient sensitivity and overlapping of signals of metabolites with those of endogenous compounds. The latter problem led to difficulties in full characterization of the above mercapturic acids and, in some cases, in relative quantitation. These could be obviated in some respects by the second application of NMR spectroscopy. In this, the metabolites were isolated from urine by TLC or HPLC and the resulting pure or partially pure metabolite was analyzed by ^{1}H-NMR at 400 MHz in an appropriate solvent [usually $CDCl_3$, $(CD_3)_2SO$ or D_2O]. Thus, metabolites present in lower concentration could be characterized, although >300 μg of material was still required. The mercapturic acids AMCC and AECC were fully identified by this less direct approach by comparison of the NMR spectra of the isolated metabolites with those of synthetic material [3, 7].

^{1}H-NMR clearly has an important role in the identification of abundant labile metabolites of non-chromophoric compounds such as formamides or acetamides, but is limited by the relative insensitivity of the technique and could not be applied to urinary metabolites arising from lower doses of the formamides in rodents or in human studies.

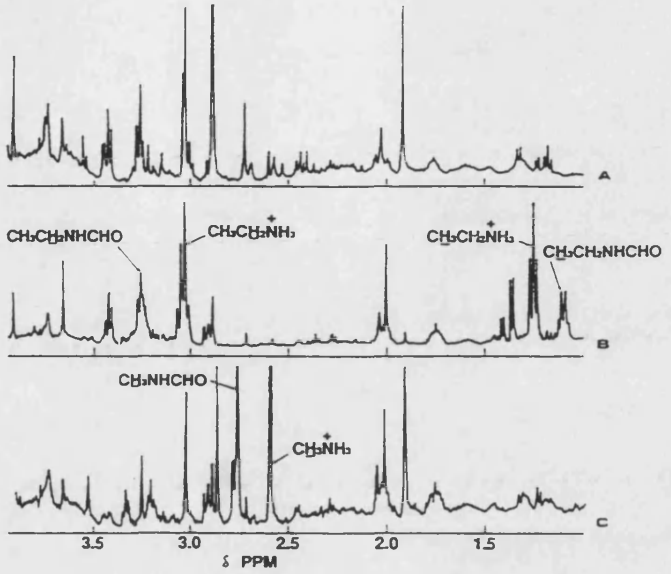

Fig. 1. Partial ^{1}H-NMR spectrum (400 MHz) of 24 h collections of urine from control mice (A), mice receiving 400 mg/kg NEF (B), and mice receiving 400 mg/kg NMF (C).

Acknowledgements

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PUBLICATION 31

Structural Studies on Bio-Active Compounds. Part 11. Molecular Modelling, Crystallographic and Biochemical Studies of the Interactions of *d,l*-α-Vinylserine with the Enzyme Serine Hydroxymethyltransferase

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Structural Studies on Bio-active Compounds. Part 11.[1] Molecular Modelling, Crystallographic, and Biochemical Studies of the Interactions of (±)-α-Vinylserine with the Enzyme Serine Hydroxymethyltransferase

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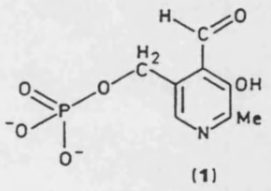
(±)-α-Vinylserine has previously been found to be a competitive inhibitor of serine hydroxymethyltransferase, an important target in anti-tumour chemotherapy. The crystal structure of (±)-α-vinylserine was determined by *X*-ray diffraction techniques, and molecular modelling was used to build the amino acid pyridoxal 5'-monophosphate Schiff's base. Molecular-mechanics calculations indicate that the reacting conformation for the cleavage of the α–β bond of the compound when bound to pyridoxal 5'-monophosphate is achievable. However, this compound, when incubated with homogeneous rabbit-liver serine hydroxymethyltransferase, was not dehydroxymethylated under any conditions, nor could any quinonoid intermediates be detected. Circular dichroism spectroscopy indicates that this molecule is not an effective enzyme inhibitor because it is not forming an imine base with the pyridoxal phosphate at the active site of the enzyme.

Serine hydroxymethyltransferase (EC 2.1.2.1), an enzyme which requires pyridoxal 5'-monophosphate (1), catalyses the interconversion of L-serine (2) and glycine (3) in the presence of tetrahydrofolate, generating a one-carbon unit in the form of N^5,N^{10}-methylenetetrahydrofolate.[2] This one-carbon unit is used in the biosynthesis of purines, pyrimidines, and methionine. The enzyme has been shown by many workers to be an apposite target for anticancer therapy.[3] While effective inhibitors for many other pyridoxalphosphate-requiring enzymes have been developed by a combination of serendipity and rational synthesis, few have been found for this enzyme. D-Fluoroalanine[4] (4) is a weak inhibitor with a K_i of 10–60 mmol dm^{-3}. L-α-Vinylserine (5) was rationally designed to be an enzyme-activated irreversible inhibitor of serine hydroxymethyltransferase, based on the knowledge that the enzyme is capable of dehydroxymethylating α-substituted serine analogues.[5] However, (±)-α-vinylserine was found to be a competitive inhibitor[5] with K_i 15.2 mmol dm^{-3}. The weakness of inhibition and the lack of time dependence may be attributable to one of three possible events occurring at the active site of the enzyme. (*a*) The compound may compete with L-serine for the active site but fail to bind *via* formation of an external aldimine. (*b*) The compound may bind to the active site pyridoxal phosphate without undergoing dehydroxymethylation. (*c*) The compound may bind and be dehydroxymethylated, but the product may be a Michael acceptor which reacts with other nucleophiles before it can react with the enzyme (or is unreactive). In order to distinguish between these hypotheses, the crystal structure of (±)-α-vinylserine has been determined, its mode of interaction with the enzyme has been predicted by molecular modelling, and the extent of the reaction with the enzyme has been investigated by biochemical techniques.



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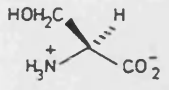

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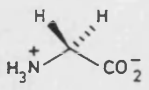

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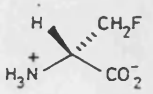

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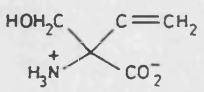

(5)

The likelihood of dehydroxymethylation was considered first. Dunathan[6] predicted that if an amino acid, bound to pyridoxal phosphate as an external aldimine, is to lose a group from its α-carbon, enhancement of the delocalisation energy of the system must occur. The greatest drop in energy will be achieved when the bond to be broken is in a plane perpendicular to the delocalised system, giving an overlap of the σ bond orbitals of the α–β bond and the π orbitals of the imine function.

Molecular modelling was used to construct the serine pyridoxal phosphate Schiff's base, as shown in Figure 1, starting from the crystal structure of pyridoxal 5'-phosphate as derived by Fujiwara.[7] When the serine N(6)–C(2) bond is rotated in increments of 10°, two relative energy minima appear (Figure 2). The conformation corresponding to one of these mimima (θ

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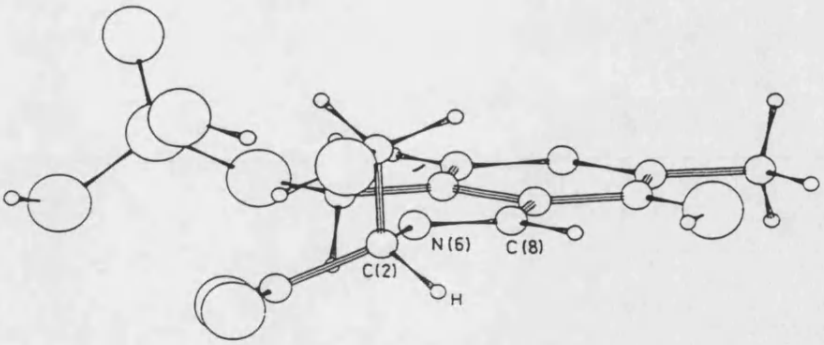

Figure 1. The modelled L-serine-pyridoxal phosphate Schiff's base.

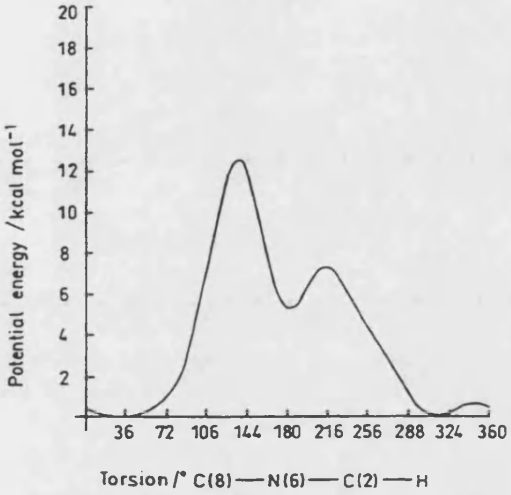

Figure 2. Effect of the rotation of the N(6)–C(2) bond of the modelled L-serine-pyridoxalphosphate Schiff's base on the potential energy of the model.

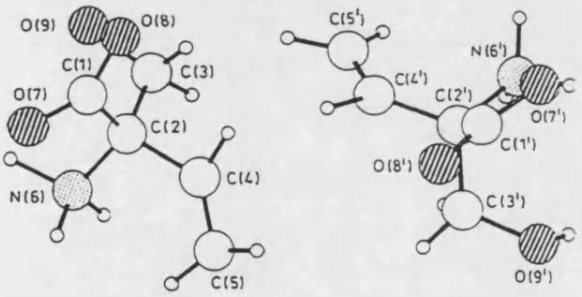

Figure 3. PLUTO drawing of the two structurally independent molecules of α-vinylserine in the asymmetric unit.

320°) has the scissile bond nearly perpendicular to the π system and is very similar to the preferred reacting conformation for the cleavage of the α–β bond.[6] Other bonds were kept fixed in this study. ^{31}P N.m.r. studies have shown that no change in the environment of the phosphate group occurs when pyridoxal phosphate binds L-serine.[8] The fixed phosphate position is responsible for the apparent high-energy interaction when the torsion angle is *ca.* 130°, but this is irrelevant to the successful construction of a model with low energy as well as favourable geometry for dehydroxymethylation.

The *X*-ray diffraction crystal structure of (±)-α-vinylserine was determined by direct methods and refined by the full-matrix least-squares method. The presented data for the zwitterionic compound confirm the inferences from spectroscopic data,[5] with bond angles and lengths being essentially as predicted. The study reveals two structurally independent but nearly superimposable molecules as shown in Figure 3. Both have the carboxy group and C(2)–N(6) bond in the same plane, with one hydrogen atom of the ammonium group being in a near-eclipsed conformation that favours intramolecular hydrogen bonding (Table 5). Corresponding bond distances in the two molecules do not differ significantly ($\Delta < 1.7\sigma$) from each other. Differences from those in (±)-serine, which was refined from neutron-diffraction data by Frey *et al.*[9] starting with the co-ordinates proposed by Shoemaker *et* al.,[10] are slight. The

Table 1. Fractional co-ordinates with estimated standard deviations in parentheses.

Atom	*x*	*y*	*z*
C(1)	0.775 3(6)	0.257 2(6)	0.669 2(6)
C(2)	0.725 8(5)	0.241 1(6)	0.777 1(6)
C(3)	0.713 3(6)	0.370 8(7)	0.835 2(7)
C(4)	0.602 0(6)	0.179 2(8)	0.705 5(8)
C(5)	0.568 8(7)	0.069 5(9)	0.726 2(10)
N(6)	0.810 7(5)	0.158 6(4)	0.893 9(5)
O(7)	0.853 4(4)	0.180 5(4)	0.673 3(4)
O(8)	0.726 8(5)	0.344 9(5)	0.584 1(5)
O(9)	0.824 3(5)	0.440 0(4)	0.887 9(5)
C(1')	0.124 6(5)	0.209 1(6)	0.115 9(6)
C(2')	0.182 3(5)	0.243 8(6)	0.270 7(6)
C(3')	0.170 4(6)	0.130 6(6)	0.354 4(6)
C(4')	0.314 2(6)	0.275 3(7)	0.323 2(7)
C(5')	0.374 3(7)	0.364 9(7)	0.408 3(8)
N(6')	0.112 3(5)	0.355 7(5)	0.288 8(5)
O(7')	0.038 0(4)	0.276 4(4)	0.034 5(4)
O(8')	0.169 7(4)	0.116 8(4)	0.080 9(4)
O(9')	0.049 0(4)	0.093 6(4)	0.312 5(5)
H(1)	0.917	0.204	0.962
H(2)	0.818	0.067	0.861
H(3)	0.772	0.155	0.975
H(4)	0.663	0.371	0.904
H(5)	0.659	0.405	0.758
H(6)	0.559	0.239	0.623
H(7)	0.494	0.062	0.640
H(8)	0.638	0.028	0.833
H(9)	0.854	0.455	0.968
H(1')	0.021	0.337	0.199
H(2')	0.142	0.350	0.401
H(3')	0.117	0.444	0.247
H(4')	0.217	0.053	0.356
H(5')	0.210	0.156	0.438
H(6')	0.367	0.216	0.261
H(7')	0.469	0.371	0.443
H(8')	0.338	0.426	0.459
H(9')	0.015	0.136	0.362

Table 2. Bond lengths/Å for non-hydrogen atoms in (±)-α-vinylserine with estimated standard deviations given in parentheses.

	Unprimed	Primed
C(1)–C(2)	1.545(8)	1.533(8)
C(1)–O(7)	1.238(7)	1.249(7)
C(1)–O(8)	1.242(7)	1.252(7)
C(2)–C(3)	1.528(9)	1.533(9)
C(2)–C(4)	1.513(9)	1.501(9)
C(2)–N(6)	1.496(8)	1.515(7)
C(3)–O(9)	1.423(8)	1.413(8)
C(4)–C(5)	1.271(11)	1.287(10)

Table 3. Bond angles/° for non-hydrogen atoms in (±)-α-vinylserine with estimated standard deviations given in parentheses.

	Unprimed	Primed
C(2)–C(1)–O(7)	117.8(5)	117.6(5)
C(2)–C(1)–O(8)	114.5(4)	117.1(5)
O(7)–C(1)–O(8)	127.7(6)	125.3(6)
C(1)–C(2)–C(3)	110.9(5)	109.7(5)
C(1)–C(2)–C(4)	108.3(4)	110.9(5)
C(1)–C(2)–N(6)	109.3(4)	107.9(5)
C(3)–C(2)–C(4)	108.9(5)	108.9(5)
C(3)–C(2)–N(6)	109.1(5)	108.0(5)
C(4)–C(2)–N(6)	110.3(5)	111.5(5)
C(2)–C(3)–O(9)	111.0(5)	113.1(5)
C(2)–C(4)–C(5)	129.0(7)	127.6(7)

Table 4. Torsion angles/° for all non-hydrogen atoms in (±)-α-vinylserine.

	Unprimed	Primed
O(7)–C(1)–C(2)–C(3)	137.7	119.15
O(7)–C(1)–C(2)–N(6)	17.4	2.2
O(8)–C(1)–C(2)–C(4)	74.4	58.6
C(1)–C(2)–C(4)–C(5)	114.1	139.8
C(3)–C(2)–C(4)–C(5)	−125.3	−99.4
O(7)–C(1)–C(2)–C(4)	−102.9	−120.2
O(8)–C(1)–C(2)–C(3)	−45.0	−61.7
O(8)–C(1)–C(2)–N(6)	−165.3	−179.1
N(6)–C(2)–C(3)–O(9)	67.0	58.9
N(6)–C(2)–C(4)–C(5)	−5.6	19.6

The estimated standard deviations range from 0.7–1.0°.

Table 5. Hydrogen bonds present in the unit cell of α-vinylserine.

Bond	Distance (Å)			Angle (°)
a–b · · · c	a–b	b–c	a · · · c	a–b–c
N(6)–H(1) · · · O(7^{I})	1.27	1.54	2.806	172
N(6)–H(2) · · · O(8^{III})	1.04	2.00	2.888	141
N(6)–H(3) · · · O(8)	1.16	1.51	2.677	177
N(6′)–H(1′) · · · O(7^{III})	1.13	1.96	2.888	136
N(6′)–H(2′) · · · O(8′)	1.10	1.84	2.910	164
N(6′)–H(3′) · · · O(9^{IV})	1.04	2.25	3.182	149
O(9)–H(9) · · · O(7^{V})	0.79	2.63	3.189	130
O(9)–H(9) · · · O(7′)	0.79	2.77	2.935	94
O(9′)–H(9′) · · · O(7′^{V})	0.92	1.97	2.803	149

Symmetry code: (I) $1+x, y, 1+z$ (II) $1-x, -y, 1-z$ (III) $-1+x, \frac{1}{2}-y, -\frac{1}{2}+z$ (IV) $1-x, 1-y, 1-z$ (V) $x, \frac{1}{2}-y, \frac{1}{2}+z$.

fractional co-ordinates for (±)-α-vinylserine are given in Table 1, and the bond lengths and angles in Tables 2 and 3 respectively. Torsion angles are given in Table 4.

Similar conformational calculations were then carried out for the modelled L-α-vinylserine–pyridoxal 5′-phosphate imine (Figure 4). The C(2)–C(4) and N(6)–C(2) bonds were rotated simultaneously and the potential energy was calculated. The data are presented in the form of a contour plot in Figure 5. Relative minima of 1 kcal mol^{-1} above the global minimum are observed at conformations (300°, 330°) and (70°, 20°). The former corresponds to the required conformation for cleavage of the α–β bond.[6] This indicates that, for a modelled system, there are no conformational energy restrictions which inhibit the dehydroxymethylation of L-α-vinylserine. It is unknown whether this conformation can occur in the active site of the enzyme. Although calculations suggest that both serine and α-vinylserine would be able to be dehydroxymethylated by serine hydroxymethyltransferase the crystallographic packing of these two racemic compounds differs markedly. Four pairs of isomers of (±)-α-vinylserine are found in the unit cell, with the enantiomers bound together by hydrogen bonds of which four have a H · · · O distance <2.3 Å, as shown in Table 5. In the crystalline state, (±)-α-vinylserine is orientated such that the polar carboxylate, alcohol and ammonium groups point towards the edge of the unit cell. The vinyl groups are directed towards the centre of the unit cell, creating alternate polar and non-polar planes extending throughout the crystal. This packing is different to that found in (±)-serine,[9,10] where the unit cell was found to contain sheets of serine stacked parallel to the [100] face.

In order to investigate whether (±)-α-vinylserine is dehydroxymethylated by the enzyme, the compound (20 mmol dm^{-3}) was incubated with homogeneous rabbit-liver cytosolic serine hydroxymethyltransferase and tetrahydrofolate over a period of 1 h. No production of methylenetetrahydrofolate could be detected under any conditions, suggesting that no cleavage of the α–β bond was occurring with this compound. This hypothesis was then further investigated using u.v. spectroscopy. The u.v. absorption spectrum of the enzyme has been studied and the intermediates produced on dehydroxymethylation have been characterised.[11] It is known that the quinonoid intermediate produced upon α–β bond cleavage has a characteristic absorption at 500 nm;[12] this can be amplified by the addition of tetrahydrofolate, which inhibits the re-protonation of the quinonoid.[13] When (±)-α-vinylserine (20 mmol dm^{-3}) was incubated with the rabbit-liver enzyme, no change in the u.v. absorption spectrum occurred and no quinonoid intermediate was detected. The effect of incubating the enzyme with glycine (5 mmol dm^{-3}) and tetrahydrofolate (0.7 mmol dm^{-3}) is shown in Figure 6.

Thus α-vinylserine is not bio-activated. This failure may imply that the compound binds to the active site of the enzyme without cleavage of the α–β bond, or the compound may not even bind to the active site. Circular dichroism (c.d.) spectroscopy was used to investigate these alternative explanations. Although pyridoxal phosphate is achiral, it has an induced optical activity when bound in the asymmetric environment of an enzyme.[14] In pyridoxal-phosphate-dependent enzymes a strong positive Cotton effect is observed due to $\pi \rightarrow \pi^*$ transitions in the aldimine chromophore.[14] The c.d. spectra of cystosolic rabbit-liver enzyme[8] and the *Escherichia coli* enzyme[15] have this absorption band present. Conversion of the internal aldimine into the external aldimine induces a change in the 320–500 nm region of the c.d. spectrum.[15] The effect of 50 mmol dm^{-3} (±)-α-vinylserine on the c.d. spectrum is shown in Figure 7. Similar results were obtained for the buffer control. The drop in the $\theta_{max.}$ is due to a dilution effect and indicates that no new aldimine bond is being formed by this compound, *i.e.* that the compound does not bind covalently to the pyridoxal phosphate in the active site of the enzyme. This is unlike the effect of the addition of 50 mmol dm^{-3} glycine, a positive control. This amino acid is forming a new aldimine bond and therefore changes the c.d. spectrum. The finding that (±)-α-vinylserine is not a substrate due to the inability of the

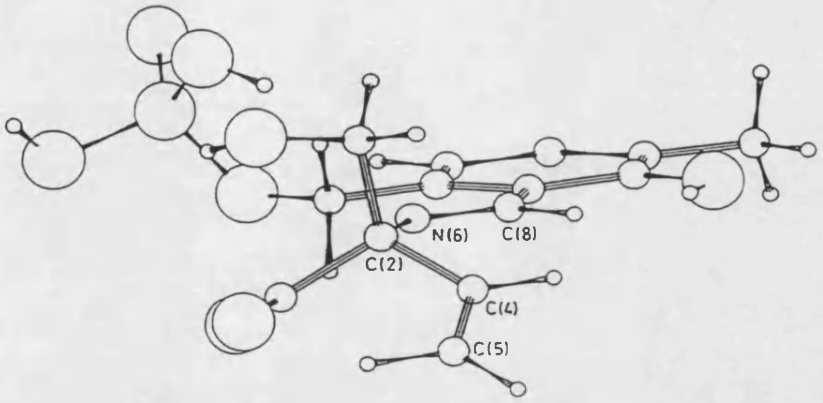

Figure 4. The modelled α-vinylserine–pyridoxal phosphate Schiff's base.

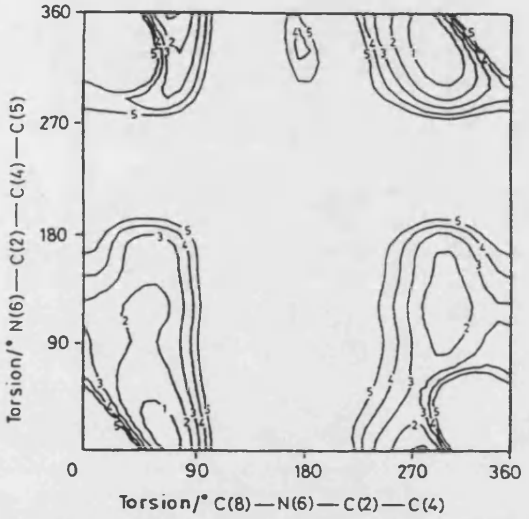

Figure 5. Effect of the simultaneous rotation of the C(2)–C(4) and N(6)–C(2) bonds of the modelled α-vinylserine–pyridoxal phosphate on the potential energy of the model (contours in kcal mol^{-1}).

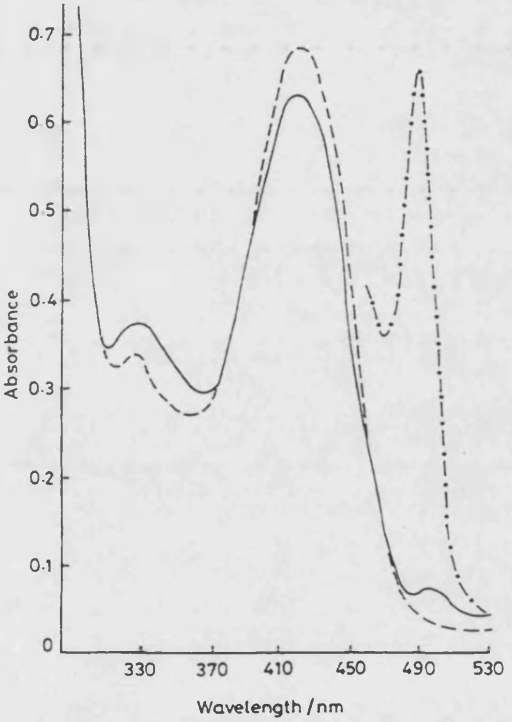

Figure 6. Effect of the addition of glycine 5 mmol dm^{-3} (– · · · –) and then tetrahydrofolate (0.7 mmol dm^{-3}) (– · – · –) on the absorption of purified rabbit-liver serine hydroxymethyltransferase (——).

compound to bind covalently to the pyridoxal phosphate at the active site of the enzyme is unexpected. It is known that other α-vinylamino acids are enzyme-activated irreversible inhibitors of pyridoxal phosphate-dependent enzymes, *e.g.* α-vinyldopa inhibits dopa decarboxylase.[16] The mechanism of transimination is well known[17] and there is no apparent reason why it should not occur with α-vinylserine, especially as it is known that serine hydroxymethyltransferase can dehydroxymethylate α-substituted serines such as α-methyl-, α-ethyl-, and α-hydroxymethylserine.[18] These amino acids have α-substituents that are approximately isosteric with the vinyl group. The reasons for the lack of binding of this compound is unknown.

This work suggests that the design and synthesis of new α-substituted serine analogues as potential inhibitors of serine hydroxymethyltransferase may not yield effective inhibitors of this enzyme. It is, however, noted that α-fluoromethylserine with its small α-substituent may be an active inhibitor and routes to the synthesis of this compound are being investigated in this laboratory.

Experimental

Preparation of (±)-α-*Vinylserine* [(±)-2-*Amino*-2-*hydroxymethylbut*-3-*enoic Acid*].—(±)-α-Vinylserine was prepared as previously described.[5] The compound (100 mg) was dissolved in water (10 cm^3) and warmed gently. Hot ethanol (20 cm^3) was added and the solution was allowed to stand for 14 days at ambient temperature. The crystalline florets of needles were separated by sonicating the suspension for 2 × 5 s. The suspension was then filtered and the crystals dried *in vacuo* for 3 days. A specimen 0.7 × 0.07 × 0.055 mm was mounted about the needle axis on a glass fibre for collection of *X*-ray diffraction data.

Crystal Data.—$C_5H_9NO_3$, M = 131.1, monoclinic, a = 12.346(3), b = 10.442(4), c = 10.763(5) Å, β = 116.70(3), V = 1 239.7 $Å^3$ (by least squares analysis of setting angles of 25 reflections, λ = 0.710 69 Å), space group $P2_1/c$, Z = 8.

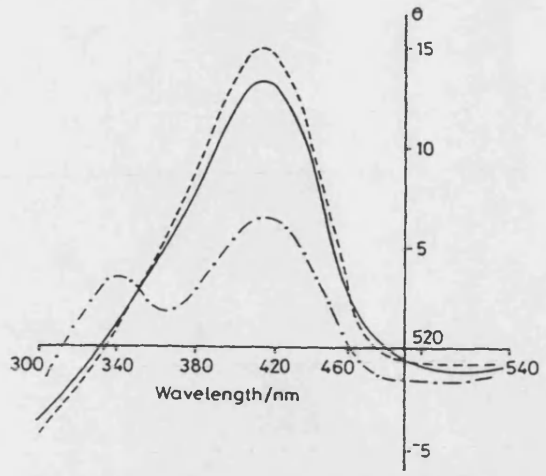

Figure 7. Effect of the addition of amino acids on the c.d. absorption spectrum of purified rabbit-liver serine hydroxymethyltransferase; (- - - -) enzyme alone; (——) effect of addition of buffer alone or α-vinylserine (50 mmol dm^{-3}); (– · – · –) effect of the addition of glycine (50 mmol dm^{-3}).

D_m = 1.41 g cm^{-3}, D_x = 1.42 g cm^{-3}, μ(Mo-K_α) = 0.75 cm^{-1}, $F(000)$ = 560.

Data Collection and Processing.—Enraf–Nonius four circle CAD-4 diffractometer, ω/2θ scans, ω scan width (1.0 + 0.35 tanθ)°, ω scan speed of 0.5–5° min^{-1} depending on intensity, graphite monochromated Mo-K_α), 4 612 reflections measured ($2 < \theta < 25°$), 2 177 unique (merging R 0.0561). 1 037 Reflections deemed observed with $|F_o| > 3\sigma(|F_o|)$. Standard deviations were calculated on the basis of counting statistics and instrument instability. Intensity and orientation monitor reflections indicated no need to correct for time-dependent instability. Maximum (sinθ)/λ reached was 0.63 with range h, k, l, 0–12, −14 to 14, and −12 to 12 respectively. No correction was made for absorption.

Structure Analysis and Refinement.—The crystal structure was solved by direct methods using the SHELX-76 program;[19] hydrogen atoms were located in difference electron density maps. Non-hydrogen atom positions and anisotropic thermal parameters together with group isotropic temperature factors for the hydrogen atoms were refined by the full-matrix least-squares technique based on the stored scattering factors (using the same temperature factors for structurally equivalent hydrogen atoms in the two independent molecules comprising the asymmetric unit).* The weighting scheme $w = (\sigma^2(|F_o|) + 0.008|F_o|^2)^{-1}$ gave satisfactory agreement analyses. The final R and R_w values are 0.0648 and 0.0693 respectively, with a goodness-of-fit ratio of S = 1.35. Molecular drawings were obtained using the PLUTO program developed by Motherwell and Clegg.[20]

Molecular Calculations.—Molecular-mechanics conformational-energy calculations were performed using the Glaxo Group Research molecular modelling system. The structure of L-serine was taken from fragments constructed from the internal database of the system. The crystal structure of pyridoxal 5′-phosphate derived by Fujiwara[7] was obtained from the Cambridge Crystallographic Database.[21] Conformational-energy calculations were performed by the summation of the individual components for the non-bonded and torsional energies.[22]

Enzyme Purification and Activity Assays.—The cytosolic rabbit-liver serine hydroxymethyltransferase was purified as previously described.[23] The purity of the enzyme was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis and the enzyme found to be a single band with the same R_F value as that previously obtained.[23] The determination of enzyme activity and the detection of the dehydroxymethylation of (±)-α-vinylserine utilised a coupled assay involving N^5,N^{10}-methylenetetrahydrofolate dehydrogenase as described by Schirch *et al.*[24]

The absorption spectra were recorded on a Cary 210 spectrophotometer, using degassed solvents, with the cuvette temperature maintained at 30 °C. The spectrum of homogeneous rabbit-liver enzyme (2 mg ml^{-1}) in 50 mmol dm^{-3} potassium phosphate pH 7.3 was recorded between 300–530 nm against buffer alone. The amino acid under test dissolved in the buffer was then added and the absorption spectrum of the enzyme was re-recorded during 1 h.

In order to amplify the production of the stabilised quinonoid intermediate, 0.7 mmol dm^{-3} tetrahydrofolate and 30 mmol dm^{-3} 2-mercaptoethanol was added and the cuvette was sealed. The absorption spectrum between 460–530 nm was re-recorded over a time period of 30 min.

C.d. spectra were recorded on a Jasco J-500C spectropolarimeter. A 1 cm pathlength cell was used with all buffers degassed prior to use. The c.d. spectrum of homogeneous cytosolic rabbit-liver enzyme (3.1 mg cm^{-3}) in 50 mmol dm^{-3} potassium phoshate pH 7.3 was recorded between 300–530 nm at a scan rate of 50 nm min^{-1}. The amino acid under test (5 mmol dm^{-3} in buffer) was added to the cell and the spectrum was re-recorded.

Acknowledgements

The assistance and helpful advice given by Drs. A. Baxter and J. D. Cocker of Glaxo Group Research is gratefully acknowledged. The molecular modelling and molecular mechanical calculations were carried out in collaboration with Drs. P. Murray-Rust and A. Tonge (Glaxo Group Research). The collaborative work undertaken at the Medical College of Virginia was made possible by the provision of a British Association for Cancer Research Travel Fellowship to S. J. B. T. and the financial support of Glaxo Group Research, the SERC and the Department of Pharmaceutical Sciences, Aston University. S. J. B. T. acknowledges the financial support of a SERC CASE award (with Glaxo Group Research). We thank Mrs. Gill Chamberlain for kindly typing this manuscript.

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* *Supplementary data* (see section 5.6.3 of Instructions for Authors, in the January issue). Thermal parameters have been deposited at the Cambridge Crystallographic Data Centre.

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PUBLICATION 32

Butylation of 2-Methylnaphthalene-2,4-dione (Menadione) by Solvent Sulpholane During Radical Methylation and Ethylation

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Synthetic Communications, **1989**, *19*, 167-172.

SYNTHETIC COMMUNICATIONS, 19(1&2), 167-172 (1989)

BUTYLATION OF 2-METHYLNAPHTHALENE-1,4-DIONE (MENADIONE) BY SOLVENT SULPHOLANE DURING RADICAL METHYLATION AND ETHYLATION

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ABSTRACT: Treatment of 2-methylnaphthalene-1,4-dione with potassium peroxydisulphate, silver nitrate and acetic or propanoic acids in aqueous sulpholane gave 3-butyl-2-methylnaphthalene-1,4-dione in addition to the expected 3-alkyl-2-methylnaphthalene-1,4-diones.

A series of 3-alkyl-2-methylnaphthalene-1,4-diones (naphthoquinones) related to menadione (1) was required for studies[1,2] on the mechanisms of metabolism and toxicity of these compounds in mammals and for further similar investigations.

Menadione was treated with potassium peroxydisulphate, silver nitrate and acetic or propanoic acids in

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aqueous sulpholane (tetrahydrothiophene-S,S-dioxide) according to the general method of Jacobsen and Torssell.[3] The major products (isolated by column chromatography) were 2,3-dimethylnaphthalene-1,4-dione (2) and 3-ethyl-2-methylnaphthalene-1,4-dione (3) respectively, arising from alkylation by methyl and ethyl radicals formed by oxidative decarboxylation of the silver carboxylates formed *in situ*. However, during the ethylation reaction which gave (3) in 68 % yield, a trace of a less polar by-product was observed by t.l.c. The same compound was obtained in 1 % yield when (1) was treated with $MeCO_2H/K_2S_2O_8/AgNO_3$ in aqueous sulpholane and, after isolation by column chromatography, was characterised as 3-butyl-2-methylnaphthalene-1,4-dione (4). Since this material was not found when sulpholane was replaced by acetonitrile as the co-solvent, according to a widely-used modification[4-6] of the original procedure, it is highly likely that the C_4 unit is derived from the sulpholane.

The generation of methyl and methylsulphinyl radicals from dimethylsulphoxide (DMSO) has been reported by Bertilsson *et al*[7] who identified (2) and 2-methylsulphonylnaphthalene-1,4-diol as products of the

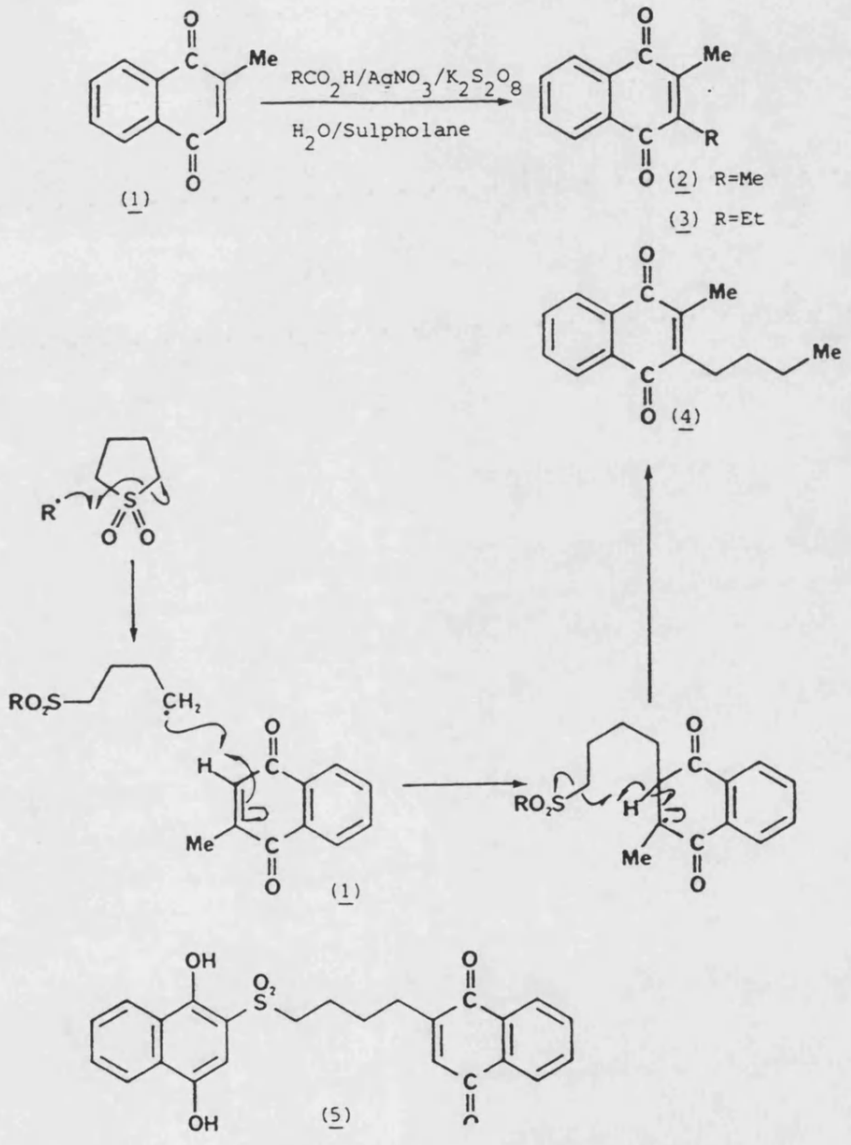
treatment of naphthalene-1,4-dione with Fenton's Reagent (H_2O_2/Fe^{2+}) in that solvent. Interestingly, replacement[7] of DMSO by tetrahydrothiophene-S-oxide gave (5) as the product arising from homolysis of the heterocycle; (4) was not reported[7] as a product. It is, therefore, likely that the butyl group of (4) does indeed arise from the sulpholane, rather than from any trace tetrahydrothiophene-S-oxide present as impurity in the solvent. A proposed mechanism for this unexpected and novel butylation is shown in the Scheme.

As butylnaphthoquinones often require very careful chromatography for separation from lower alkyl homologues, these observations identify the source of these contaminants in the desired products of radical alkylation of naphthoquinones. Sulpholane is, therefore, indicated as an inappropriate solvent for these synthetic procedures.

EXPERIMENTAL

NMR spectra were obtained in $CDCl_3$ at 60 MHz using a Varian EM360A spectrometer and a VG Micromass 12B instrument furnished the mass spectra. Melting points are corrected.

SCHEME


RCO2H/AgNO3/K2S2O8
H2O/Sulpholane
Me
R
(1)
(2) R=Me
(3) R=Et
(4)
(5)
OH
OH
RO2S
CH2
H

2,3-Dimethylnaphthalene-1,4-dione (2) and 3-butyl-2-methylnaphthalene-1,4-dione (4). 2-Methylnaphthalene-1,4-dione (1: 6.88 g, 40 mmol), acetic acid (3.0 g, 50 mmol) and silver nitrate (2.0g, 11.8 mmol) were heated to 80°C in a mixture of tetrahydrothiophene-S,S-dioxide (sulpholane: 80ml) and water (120 ml). Potassium peroxydisulphate (22.0g, 81.5 mmol) was added during 40 min at 80°C. The mixture was poured onto ice (440 g) and the yellow gum was collected and chromatographed (silica gel: ethyl acetate: hexane 1:4) to give 3-butyl-2-methylnaphthalene-1,4-dione (4: 80 mg, 1 %) from the faster running fraction, yellow needles MPt 67-8°C (lit.[6] MPt 68-9°C), δ 0.95 (3H, t, J 7 Hz, $ArCH_2CH_2CH_2C\underline{H}_3$), 1.4 (4H, m, $ArCH_2C\underline{H}_2C\underline{H}_2CH_3$), 2.25 (3H, s, $ArCH_3$), 2.60 (2H, m, Ar $C\underline{H}_2CH_2CH_2CH_3$), 7.6-7.9 (4H, m, ArH); m/z 228 (M^+). From slightly later running fractions was obtained 2,3-dimethylnaphthalene-1,4-dione (2: 2.10 g, 28 %) identical with material previously reported[1] from the radical dimethylation of naphthalene-1,4-dione.

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PUBLICATION 33

Synthesis of Peptides Containing *S*-(*N*-Alkylcarbamoyl)cysteine Residues, Metabolites of *N*-Alkylformamides in Rodents and in Man

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Journal of Organic Chemistry, **1989**, *54*, 2940-2949.

Synthesis of Peptides Containing *S*-(*N*-Alkylcarbamoyl)cysteine Residues, Metabolites of *N*-Alkylformamides in Rodents and in Humans

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Received August 24, 1988

Hydrochloride salts of *S*-(*N*-methylcarbamoyl), *S*-(*N*-ethylcarbamoyl), and *S*-(*N*,*N*-dimethylcarbamoyl) derivatives of cysteine, *N*-acetylcysteine, and cysteinylglycine have been prepared. *S*-(*N*-Methylcarbamoyl)glutathione hydrochloride has also been synthesized. Protecting groups for amino and carboxylic acid functions were selected for their ability to solubilize the peptides in dichloromethane in which solvent the thiols were treated with alkyl isocyanates and with *N*,*N*-dimethylcarbamoyl chloride. Removal of *S*-(amidomethyl) protecting groups using mercury(II) acetate was found to cause some loss of *N*-(*tert*-butoxycarbonyl) groups. Elimination of disulfide was evident during coupling of disulfide derivatives of cysteine using mixed anhydride methods but not with a carbodiimide coupling agent. Mixed disulfide protections were reductively cleaved by propane-1,3-dithiol. Many of the deprotected *S*-carbamoyl amino acids and peptides are metabolites of the corresponding *N*-alkylformamides in rodents and in humans.

N-Substituted formamides have a variety of biological activities, both beneficial and adverse. *N*-Methylformamide (NMF; **1a**) has been found to be an antitumor agent in experimental systems[2] and also to be an hepatotoxin,[3,4] whereas *N*-ethylformamide (**1b**) has little or no anticancer activity[2] but is also toxic to the liver.[5] *N*,*N*-Dimethylformamide (DMF; **1c**), however, displays both of these effects only weakly in rodents.[2,5,7] We have recently shown that the two secondary amides are metabolized to the corresponding mercapturic acids **5a**,**b** in mice[6] and that this metabolic pathway (Scheme I) is implicated[5,8,9] in the hepatotoxicity of **1a**,**b**. A mass spectrometric study[9] has also enabled the characterization of the glutathione derivative **2a** as a metabolite of **1a** in mice. *N*-Acetyl-*S*-(*N*-methylcarbamoyl)cysteine (**5a**) has also been detected[7] in the urine of mice and humans exposed to **1c**, with an apparent parallel between the amount excreted and the extent of hepatotoxicity. Selective oxidation of the formyl group of *N*-methylformamide has been reported rarely in

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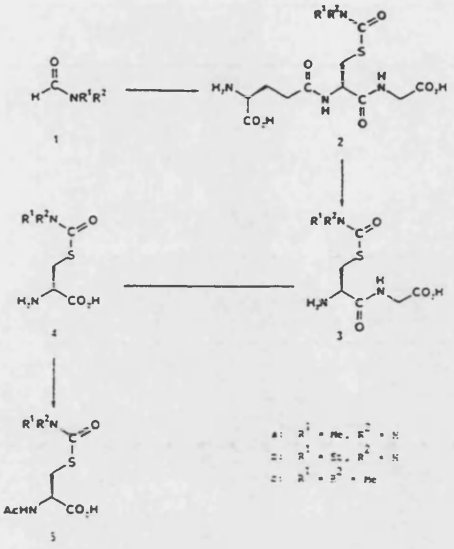
Scheme I. Partial Proposed Routes of Metabolism of Formamides in the Mouse



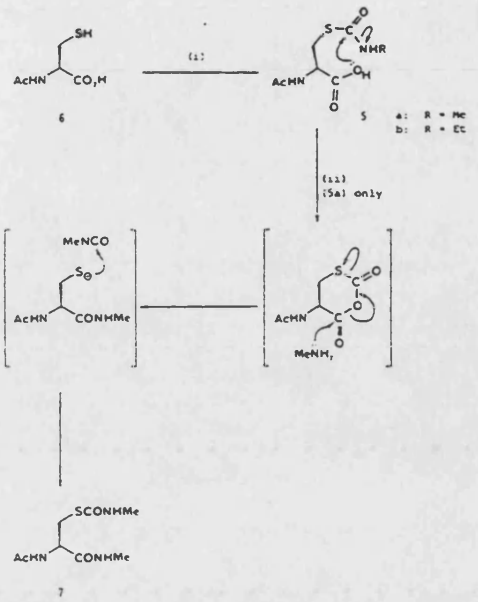
[a] Compounds 2a and 5a,b have been shown to be metabolites.

the chemical literature, oxidants being limited to transition metal ions,[12] ketones (under drastic conditions in the Leukart reaction),[13] and elemental selenium;[14] the latter gives a carbamoylating intermediate possibly analogous to that formed in vivo. The synthesis of the proposed metabolic intermediates and the *N,N*-dimethyl analogues 2–5 was therefore of importance for the study of their chemical, biochemical, and toxicological properties.

The preparation of *S*-(*N*-ethylcarbamoyl)cysteine (4b) has been reported by Guttmann[15] who used it as an S-protected cysteine during syntheses of glutathione (21) and oxytocin. In our laboratory, however, neither cysteine nor its N-acetylated analogue 6 reacted smoothly with methyl isocyanate in DMF according to this method.[15] Treatment of *N*-acetylcysteine (6) with methyl isocyanate or with ethyl isocyanate in anhydrous pyridine at 0 °C gave 5a,b, respectively, in good yield.[8] Higher temperatures (>15 °C) led to exclusive formation of the corresponding *N*-methylamide 7 from 6 and methyl isocyanate. A route of the type shown in Scheme II is likely to be involved, although direct formation of N-substituted amides by treatment of carboxylic acids with isocyanates has been reported.[16]

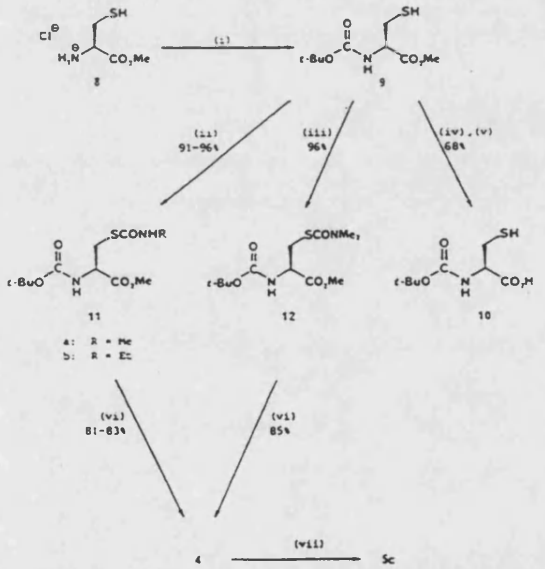
Owing to the reactivity of isocyanates and dimethylcarbamoyl chloride with polar solvents, protecting groups for amino and carboxyl functions of 6 and cysteine were sought, which would confer good solubility in the less polar and nonnucleophilic organic solvents. Facile removal upon mild treatment with acid was also required in view of the lability of thiocarbamates to base.[8,15] A suitably protected cysteine derivative 9 was prepared in high yield by the selective *tert*-butoxycarbonylation of cysteine methyl ester 8 with di-*tert*-butyl dicarbonate in dichloromethane in the presence of a tertiary amine base (Scheme III). No reaction of the thiol was observed under these conditions.

Scheme II. Reactions of *N*-Acetylcysteine (6) with Alkyl Isocyanates in Pyridine[a]



[a] (i) RNCO/pyridine/0 °C; (ii) MeNCO/pyridine/25 °C.

Scheme III. Syntheses of *S*-(*N*-Alkylcarbamoyl)cysteines 4 and the *N*-Acetyl Analogue 5c[a]



[a] (i) $(Bu^tOCO)_2O/Et_3N/CH_2Cl_2$; (ii) RNCO/($Pr^i_2NEt$ or Et_3N)/CH_2Cl_2; (iii) Me_2NCOCl/pyridine/CH_2Cl_2; (iv) NaOH/MeOH; (v) H^+; (vi) 9 M aqueous HCl; (vii) Ac_2O/pyridine (4c only).

The resulting N-protected ester 9 could be hydrolyzed smoothly with methanolic sodium hydroxide to *N*-(*tert*-butoxycarbonyl)cysteine (10). Now suitably protected and solubilized, 9 was carbamoylated smoothly by using the appropriate isocyanate or dimethylcarbamoyl chloride in dichloromethane. Deprotection of the resulting *S*-carbamoyl compounds 11a,b and 12 to the desired cysteine derivatives 4 was effected by dissolution in concentrated hydrochloric acid for a prolonged period, a procedure to which the thiocarbamate proved largely inert. Subsequent acetylation of the *N,N*-dimethyl compound 4c with acetic

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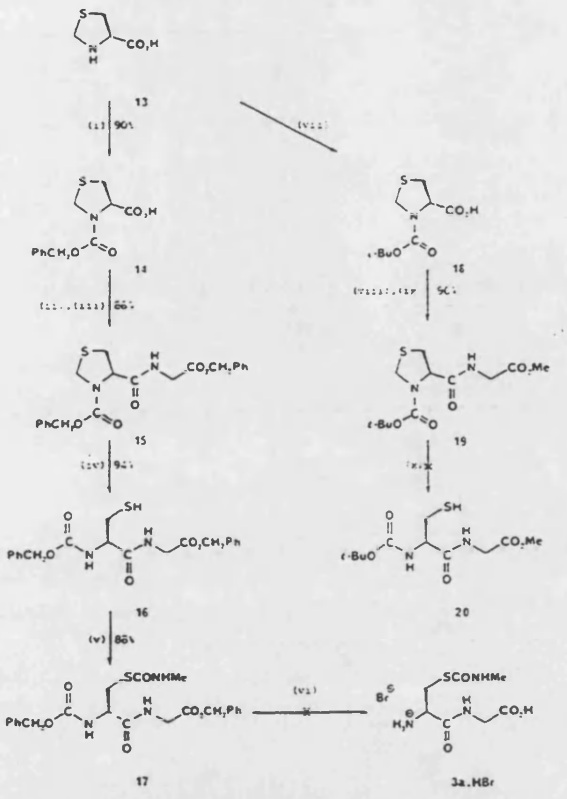
Scheme IV. Attempted Approaches to *S*-(*N*-Alkylcarbamoyl)cysteinylglycines from 1,3-Thiazolidine-4-carboxylic Acid (13)[a]



[a] (i) $PhCH_2OCOCl/KOH/H_2O$; (ii) dicyclohexylcarbodiimide/CH_2Cl_2; (iii) $GlyOCH_2Ph$-HOTs/Pr^i_2NEt/CH_2Cl_2; (iv) $Hg(OAc)_2$/$HOAc/H_2O$/60 °C; (v) $MeNCO/Pr^i_2NEt/CH_2Cl_2$; (vi) HBr/HOAc; (vii) $(Bu^tOCO)_2O/NaOH/Et_2O/H_2O$; (viii) $Bu^tOCOCl/Et_3N/CH_2Cl_2$; (ix) GlyOMe-HCl/Et_3N/CH_2Cl_2; (x) $Hg(OAc)_2$/various conditions.

anhydride in pyridine gave the mercapturic acid 5c.

For the syntheses of the dipeptide series of compounds 3, initial approaches to a suitable amino- and carboxy-protected and solubilized cysteinylglycine centered on the preparation of the *N*-(benzyloxycarbonyl)cysteinylglycine esters 16 and 17. Protection of the thiol moiety with an amidomethyl group was an attractive proposition as such groups are reported[17] to be labile to mild acid in the presence of mercuric or cadmium ions, conditions to which *N*-(benzyloxycarbonyl) groups and carboxylic esters are stable. An intramolecular version of an *S*-(amidomethyl)-protected cysteine is available via acylation of 1,3-thiazolidine-4-carboxylic acid (thiaproline; 13) (Scheme IV). Both *N*-(benzyloxycarbonyl)thiazolidinecarboxylic acid 14 and the *N*-(*tert*-butoxycarbonyl) analogue 18 proved to be excellent substrates for the carbodiimide method of peptide coupling; *N*-[[*N*-(benzyloxycarbonyl)-1,3-thiazolidin-4-yl]carbonyl]glycine benzyl ester (15) and the methyl ester 19 of the corresponding *N*-(*tert*-butoxycarbonyl) dipeptide were prepared in high yield. Treatment of the benzyl compound 15 with mercury(II) ions in warm aqueous acetic acid caused deprotection at sulfur to give the thiol 16, whereas the *N*-(*tert*-butoxycarbonyl) group of 19 was not stable to any

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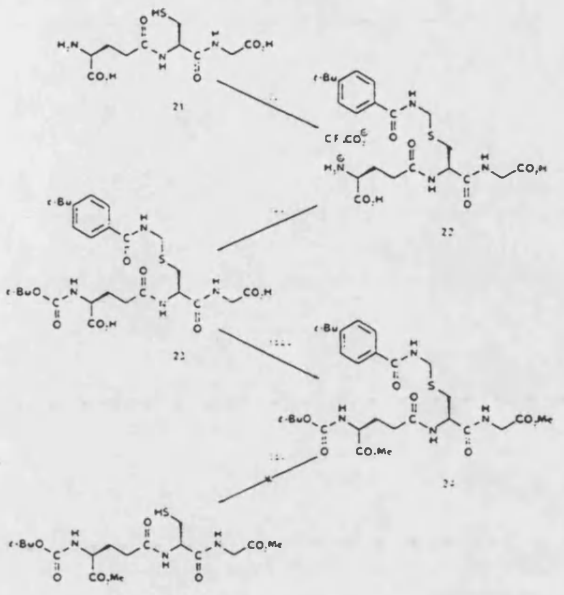
Scheme V. Loss of the *N*-(*tert*-Butoxycarbonyl) Group during Removal of an *S*-(Amidomethyl) Group from Glutathione[a]



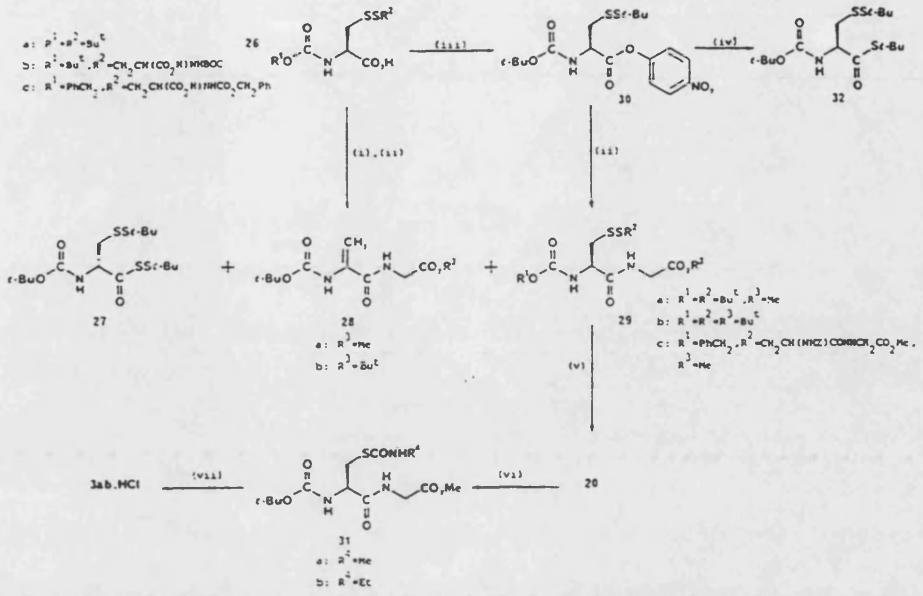
[a] (i) 4-*tert*-Butyl-*N*-(hydroxymethyl)benzamide/CF_3CO_2H;[18] (ii) $(Bu^tOCO)_2O/Et_3N/Et_2O/H_2O$; (iii) CH_2N_2/Et_2O; (iv) $Hg(OAc)_2$/MeOH or $HOAc/H_2O$.

similar conditions which would hydrolyze the "*S*-amidomethyl" function; thus 20 is unavailable by this route (Scheme IV). The dibenzyl compound 16 reacted with methyl isocyanate to give the *S*-(*N*-methylcarbamoyl)-protected dipeptide 17 (Scheme IV). However, the *S*-carbamoyl moiety was found not to be stable to hydrogen bromide in acetic acid in this case, in contrast to the analogous deprotections reported to be useful by Guttmann.[15] Only traces of the correct *S*-(*N*-methylcarbamoyl)cysteinylglycine (3a) could be detected by mass spectrometry (cesium ion liquid secondary ionization MS) in the crude product mixtures. In the tripeptide series, a sample[18] of the S-protected glutathione 22 was *tert*-butyloxycarbonylated at nitrogen, giving 23, and the dimethyl ester 24 was formed (Scheme V). Again, no conditions could be found in which the *N*-BOC function outlasted the *S*-amidomethyl group. Alternative methods of removal of amidomethyl protecting groups either destroyed the *N*-BOC group (Cd^{2+}/acetic acid) or were considered to be inappropriate.[19,20]

A conceptually different form of temporary inactivation of thiols as irreversible nucleophiles is to form either symmetrical or mixed disulfides from which the thiol can be unmasked by reduction. Owing to some difficulties in separating the coformed *N*,*N*′-dicyclohexylurea from the products from the carbodiimide couplings above, a cleaner coupling system was investigated for the reactions of the symmetrical disulfides, *N*,*N*′-bis(*tert*-butoxycarbonyl)-cystine (26b) and *N*,*N*′-bis(benzyloxycarbonyl)cystine (26c), and the unsymmetrical disulfide *N*-(*tert*-butoxy-

(18) Addison, S. J.; Cunningham, B. D. M.; Gate, E. N.; Shah, P. Z.; Threadgill, M. D. *J. Chem. Soc., Perkin. Trans. 1* 1985, 75.
(19) Moroder, L.; Marchiori, F.; Borin, G.; Schoffone, E. *Biopolymers* 1973, *12*, 493.
(20) Fontana, A. *J. Chem. Soc., Chem. Commun.* 1975, 976.
(21) Anderson, G. W.; Zimmerman, J. E.; Callaghan, F. M. *J. Am. Chem. Soc.* 1966, *88*, 1338.

Scheme VI. Preparation of *S*-(*N*-Alkylcarbamoyl)cysteinylglycines 3 and Elimination of RSS⁻ during Mixed-Anhydride Coupling[a]



[a] (i) $Bu^tOCOCl/(Pr^i_2NEt$ or $Et_3N)/CH_2Cl_2$; (ii) $X^-H_3N^+CH_2CO_2R^3/Pr^i_2NEt$ or Et_3N/CH_2Cl_2; (iii) dicyclohexylcarbodiimide/4-nitrophenol/CH_2Cl_2 (**26a** only); (iv) $Bu^tSH/Pr^i_2NEt/CH_2Cl_2$; (v) $HSCH_2CH_2CH_2SH/Pr^i_2NEt$/tetrahydrofuran; (vi) $R^4NCO/Pr^i_2NEt/CH_2Cl_2$; (vii) 9 M aqueous HCl.

carbonyl)-*S*-(*tert*-butylthio)cysteine (**26a**) (Scheme VI). The "mixed-anhydride" method has been widely used to form amide bonds and mixed carboxylic–carbonic anhydrides derived from isobutyl chloroformate have been reported[19] to be particularly effective. In the present case, *N,N'*-di-BOC-cystine **26b** was treated with isobutyl chloroformate and glycine methyl ester at ambient temperature. The sole isolable product (in moderate yield) was characterized as the protected dehydroalanylglycine **28a**. This elimination also took place to a minor extent (giving **28b**) during the coupling of the unsymmetrical disulfide **26a** with glycine *tert*-butyl ester, as shown by NMR of the crude product. Unwanted elimination of an activated sulfur group from a cysteinyl peptide has been reported[22] to occur, for example, when the *S*-(dimethylthionophosphino) protecting group is employed. However, the elimination of an apparently unactivated disulfide during a mixed-anhydride coupling procedure is undocumented. The mechanism of the process is unclear. Also formed during the reaction of **26a** with glycine *tert*-butyl ester, isobutyl chloroformate, and tertiary amine base were the expected product **29b** and a low yield of an unstable oily material tentatively characterized as the *S*-(*tert*-butylthio) thioester **27**. This compound has similar, but distinct, spectroscopic properties from the analogous *tert*-butyl thioester **32** prepared from the 4-nitrophenyl ester **30** (see below). Thioester **27** may well result from acylation of *tert*-butyl disulfide anion by the mixed anhydride, and its formation implies that the elimination of Bu^tSS^- is taking place while the anhydride is still unreacted with the glycine ester and possibly before the amino acid ester is added. In contrast, the coupling of **26a** and *N,N'*-bis(benzyloxycarbonyl)cystine (**26c**) with glycine methyl ester by this mixed-anhydride method gave only the protected dipeptides **29a,c**. During some experiments, however, the crude products were shown by NMR to be contaminated with the corresponding 2-methylprop-1-yl esters. Compound **29c** had properties identical with those described by Zahn and Schmidt[23] and by Zervas et al.[24] but not to those claimed by Dadič et al.[25] for this material. A more reliable synthesis of the potentially useful **29a**, with *N*-, S-, and O-protecting groups separately removable under conditions of anhydrous acid, reduction, and aqueous base, was therefore sought.

N-BOC-*S*-(*tert*-butylthio)cysteine (**26a**) was coupled in good yield with 4-nitrophenol by the carbodiimide method to give the "active" ester **30** (Scheme VI). This ester reacted smoothly with 2-methylpropane-2-thiol to give the thioester **32** alluded to above. The reaction with glycine esters was similarly rapid and facile, affording the protected dipeptide methyl and *tert*-butyl esters **29a,b** in almost quantitative yield. Of these dipeptide esters, **29a** is the more synthetically useful, being the precursor of S-substituted cysteinylglycines and, after further elaboration, of *S*-(*N*-methylcarbamoyl)glutathione (**2a**).

In the approach to **3a,b**, selective reduction of the unsymmetrical disulfide moiety of **29a** was required. The reductive removal of mixed disulfide protecting groups during peptide synthesis has been reported to be effected by sodium borohydride (although no details of yield or method were given)[26] and by treatment with thiophenol.[27] In the present work, reduction of **29a** by treatment with ethanolic sodium borohydride was not successful and proceeded only very poorly with thiophenol. A dithiol, such as propane-1,3-dithiol, should be a more effective reagent for this purpose. After initial intermolecular di-

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(23) Zahn, H.; Schmidt, G. *Justus Liebigs Ann. Chem.* **1970**, *731*, 91.

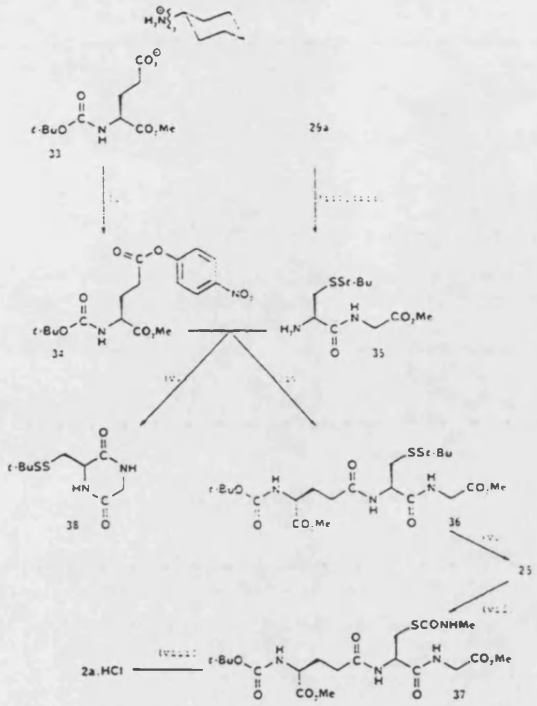
(24) Zervas, L.; Photaki, I.; Ghelis, N. *J. Am. Chem. Soc.* **1963**, *85*, 1337.

(25) Dadič, M.; Fleč, D.; Markovic-Prpič, A. *Croat. Chim. Acta* **1961**, *33*, 73.

(26) Wünsch, E.; Spangenberg, R. In *Peptides 1969*; Scoffone, E., Ed.; North Holland, 1971.

(27) Inukai, N.; Nakano, K.; Murakami, M. *Bull. Chem. Soc. Jpn.* **1967**, *40*, 2913.

Scheme VII. Synthesis of *S*-(*N*-Methylcarbamoyl)glutathione (2a)[a]



[a] (i) Dicyclohexylcarbodiimide/4-nitrophenol/CH_2Cl_2; (ii) CF_3CO_2H; (iii) base; (iv) Pr^i_2NEt/4-(dimethylamino)pyridine/tetrahydrofuran; (v) Pr^i_2NEt/tetrahydrofuran; (vi) $HSCH_2CH_2CH_2SH$/Pr^i_2NEt/tetrahydrofuran; (vii) MeNCO/Et_3N/CH_2Cl_2; (viii) 9 M aqueous HCl.

sulfide exchange (with exogenous thiolate presumably attacking the less sterically hindered cysteinyl sulfur rather than that adjacent to the *tert*-butyl group), a subsequent intramolecular disulfide exchange would lead to the required *N*-BOC-cysteinylglycine with a free thiol for further reaction with carbamoylating agents. This process was, indeed, found to be satisfactory, in that treatment of the protected dipeptide **29a** with an excess of propane-1,3-dithiol in tetrahydrofuran gave the dipeptide thiol **20** in good yield. The rate of the process was considerably enhanced by addition of a hindered tertiary amine to aid intermediate formation of the appropriate thiolate anions. The byproduct, 1,2-dithiacyclopentane, has been reported[28-30] to be unstable and to give rise to intractable insoluble polymers, but no such degradation was evident during any of these reductive deprotection steps. The dipeptide thiol **20** reacted with methyl and ethyl isocyanates in the presence of catalytic tertiary amine base to give the thiocarbamates **31a,b**, respectively. Deprotection of the N- and C-terminals of these dipeptides was achieved in a "one-pot" process involving dissolution of **31** in concentrated aqueous hydrochloric acid. Rapid evolution of gaseous carbon dioxide and 2-methylpropene indicated that the removal of the *tert*-butoxycarbonyl group was complete within 5 min, whereas hydrolysis of the methyl ester took several hours (as shown by proton NMR monitoring of an experiment with 35% deuterium chloride in deuterium oxide as the hydrolytic medium). Evaporation of the reagents gave the unprotected *S*-(*N*-alkylcarbamoyl)cysteinylglycines (**3a,b**) as the hydrochloride salts.

The selective deprotection (using trifluoroacetic acid) of the amino function of **29a** was exploited in the successful synthetic approach to the glutathione derivative **2a** (Scheme VII). The evaporation residue comprised the trifluoroacetate salt of the amine **36** from which the free nucleophile could be liberated for reaction with an appropriate *N*- and α-carboxyl-protected glutamic acid bearing an activated γ-carboxyl function. Following the success of the corresponding protection and activation strategies in the dipeptide series, the novel *N*-(*tert*-butoxycarbonyl)glutamic acid α-methyl γ-(4-nitrophenyl) diester **34** was the chosen electrophile for the coupling reaction and was synthesized as follows. *N*-BOC-glutamic acid α-methyl ester dicyclohexylammonium salt (**33**) was prepared generally by the method of Schröder and Klieger.[31] The γ-carboxylic acid moiety was then coupled with 4-nitrophenol by the dicyclohexylcarbodiimide method, giving the diester **34** in excellent yield after chromatography. The correct regioisomeric identity of **34** as being the α-methyl γ-(4-nitrophenyl) diester was confirmed, after satisfactory elemental and spectroscopic analyses, by the significant difference between the melting point of this material and the reported[32] melting point of *N*-(*tert*-butyloxycarbonyl)glutamic acid α-(4-nitrophenyl) γ-methyl diester. The reaction of the "active ester" **34** with **35** was efficient but was markedly slower than the acylation of glycine esters by the protected cysteine 4-nitrophenyl ester **30**, probably owing to the bulky nature of the dipeptide nucleophile. Catalysis by 4-(dimethylamino)-pyridine was required, since its absence led to lower yields of **36** and formation of the diketopiperazine **38**. Reductive deprotection of this tripeptide **36** with propane-1,3-dithiol was again effective, and the thiol **25** was obtained in good yield. As in the case of protected cysteine **9** and the protected cysteinylglycine **20**, treatment with methyl isocyanate in dichloromethane in the presence of *N,N*-diisopropylethylamine gave high yields of the corresponding *N*-BOC-*S*-(*N*-methylcarbamoyl)glutathione dimethyl ester **37**. Deprotection of the amino group and the carboxylic acids was again carried out in high yield with aqueous acid. Using deuterated solvent and reagent, the reaction of the *S*-(*N*-methylcarbamoyl)-protected glutathione **37** with acid was monitored by ^1H NMR spectroscopy, revealing that, as expected, the *tert*-butyl and *N*-carboxy groups were eliminated immediately upon dissolution. The hydrolyses of the methyl esters were seen to be almost complete in 20 min and 20 h, respectively, although it is not clear which ester of the intermediate protonated S-substituted glutathione diester is the more labile.

Optical rotations were measured for 12 representative compounds. In no case was complete racemization observed during the various chemical transformations. Significant partial loss of stereochemical integrity is also unlikely, as there was no evidence of other diastereoisomers in the NMR spectra of the substituted glutathiones **2a**, **25**, and **36**.

Evaluation of the biochemical and biological properties of the synthetic *S*-(*N*-alkylcarbamoyl)cysteines and peptides is in progress and will be described elsewhere. Interestingly, the free base of *S*-(*N*-ethylcarbamoyl)cysteine together with the analogous *S*-[*N*-(2-chloroethyl)carbamoyl]cysteine have been reported to have antitumor,[33]

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(29) Calvin, M.; Barltrop, J. A. *J. Am. Chem. Soc.* **1952**, *74*, 6153.
(30) Barltrop, J. A.; Hayes, P. M.; Calvin, M. *J. Am. Chem. Soc.* **1954**, *76*, 4348.
(31) Schröder, E.; Klieger, E. *Justus Liebigs Ann. Chem.* **1964**, *673*, 196.
(32) Levy, D.; Carpenter, F. H. *Biochemistry* **1967**, *6*, 3559.

antimicrobial,[34] and cytotoxic[33] properties. In addition to the preparation of the thiocarbamates **2a**, **3a,b**, **4**, and **5**, the successful synthetic route described above involves the preparation, in good yield, of the glutathione derivative **40**, with separately removable protecting groups for amino, thiol, and carboxyl functions, and furnishes a potentially highly useful intermediate in the chemical synthesis of of S-substituted glutathiones. These compounds are involved[35-38] in the metabolic activation or detoxification of many xenobiotic organic compounds.

Experimental Section

IR spectra were recorded as liquid films (except where noted) with Perkin–Elmer 1310 or Philips PU9516 spectrometers. NMR spectra were obtained (in $CDCl_3$ except where noted otherwise) at 60 MHz with Varian EM360A or JEOL PMX60SI spectrometers, at 300 MHz with Varian XL300 or Bruker AC300 spectrometers, and at 400 MHz with a Bruker WH400 instrument. Optical rotations were measured with an Optical Activity Ltd. AA-10 polarimeter. Electron impact mass spectra were furnished by VG Micromass 12B and ZAB-E instruments. Cesium ion promoted liquid matrix secondary ionization mass spectra (LSIMS) were obtained with a Kratos MS-50S mass spectrometer equipped with a 23-kG magnet and a postacceleration detector operating at −10 kV. Samples were dissolved in a glycerol matrix containing HCl (to increase the intensity of MH^+ species), and ionization was achieved by bombardment with a 1.0-μA primary beam of Cs^+ ions.[39] Melting points are corrected. Reactions were carried out at ambient temperature except where indicated otherwise. Organic solutions were dried by treatment with anhydrous Na_2SO_4 and filtration. Solvents were evaporated under reduced pressure. All chiral amino acids were of the L configuration. THF refers to tetrahydrofuran.

***S*-(*N*-Methylcarbamoyl)glutathione Hydrochloride (2a).** Compound **25** (435 mg, 1 mmol) was stirred for 3 days with methyl isocyanate (285 mg, 5 mmol) and *N,N*-diisopropylethylamine (129 mg, 1 mmol) in CH_2Cl_2 (10 mL). The solution was washed with ice-cold aqueous H_2SO_4 (1 M), H_2O, and saturated aqueous NaCl before being dried. Evaporation of the solvent afforded **37** (400 mg, 81%) as a colorless gum: IR 3300, 1720, 1700, 1680 cm^{-1}; NMR δ 1.43 [9 H, s, $C(CH_3)_3$], 1.9–2.5 (4 H, m, Glu β-CH_2 and Glu γ-CH_2), 2.83 (3 H, d, J = 5 Hz, NCH_3), 3.3 (2 H, m, Cys β-CH_2), 3.74 (6 H, s, 2 OCH_3), 4.00 (2 H, d, J = 5 Hz, Gly CH_2), 4.24 (1 H, m, Glu α-H), 4.75 (1 H, m, Cys α-H), 5.75 (1 H, d, J = 7 Hz, Glu NH or Cys NH), 7.3–7.7 (2 H, m, 2 NH), and 8.41 (1 H, ca. q, J = ca. 5 Hz, $NHCH_3$); mass spectrum, m/z 335 [(M − C_4H_8 − CO_2 − MeNCO)$^+$], 304 [(M − C_4H_8 − CO_2 − $NHCH_2CO_2CH_3$)$^+$], 247, 57 (100%). This material (246 mg, 0.5 mmol) was treated with 35% DCl in D_2O (2 mL) at ambient temperature for 4 days before excess reagent was evaporated. H_2O (5 mL) was added. The gummy evaporation residue was triturated with acetone and then with anhydrous THF to afford **2a** (160 mg, 65%) as a very hygroscopic pale buff solid of indefinite melting point (dec) which did not give a satisfactory microanalysis but was shown to be >95% pure by NMR: IR (Nujol) 2900–2600, 1710, 1690 cm^{-1}; NMR (D_2O) δ 2.10 (2 H, m, Glu β-CH_2), 2.45 (2 H, m, Glu γ-CH_2), 2.75 (3 H, s, NCH_3), 3.15 (1 H, dd, J = 14 Hz, J = 7 Hz) and 3.40 (1 H, dd, J = 14 Hz, J = 4 Hz) (Cys β-CH_2), 3.75 (1 H, t, J = 7 Hz, Glu α-H), 3.95 (2 H, s, Gly CH_2), and 4.50 (1 H, m, Cys α-H).

***N*-[*S*-(*N*-Methylcarbamoyl)cysteinyl]glycine Hydrochloride (3a).** Compound **31a** (200 mg, 573 μmol) was treated with aqueous HCl (9 M) for 1 week. Trituration of the gummy evaporation residue with acetone afforded **3a** (143 mg, 92%) as an hygroscopic white powder which decomposed on heating at >70 °C: NMR (D_2O) δ 2.70 (3 H, s, NCH_3), 3.2–3.35 (2 H, m, Cys β-CH_2), 4.00 (2 H, s, Gly CH_2), and 4.15 (1 H, m, Cys α-H); mass spectrum (FAB), m/z 236 [(M + H)$^+$]. Anal. Calcd for $C_7H_{14}ClN_3O_4S$: C, 30.95; H, 5.2; N, 17.45. Found: C, 30.65; H, 5.5; N, 17.25.

***N*-[*S*-(*N*-Ethylcarbamoyl)cysteinyl]glycine Hydrochloride (3b).** Compound **31b** was treated with aqueous HCl, as for the preparation of **3a** above, to give **3b** (83%) as an hygroscopic white powder which decomposed on heating at >70 °C: NMR (D_2O) δ 1.15 (3 H, t, J = 7 Hz, CH_2CH_3), 2.90 (2 H, q, J = 7 Hz, NCH_2CH_3), 3.2–3.4 (2 H, m, Cys β-CH_2), 4.00 (2 H, s, Gly CH_2), and 4.20 (1 H, m, Cys α-H); mass spectrum (FAB), m/z 250 [(M + H)$^+$]. Anal. Calcd for $C_8H_{16}ClN_3O_4S$: C, 33.65; H, 5.65; N, 14.7. Found: C, 33.35; H, 5.9; N, 14.5.

***S*-(*N*-Methylcarbamoyl)cysteine Hydrochloride (4a).** Ester **11a** (1.20 g, 4.1 mmol) was treated with aqueous HCl (9 M; 25 mL) for 1 week. The gummy evaporation residue was triturated with propan-2-ol (30 mL) to give a white solid (100 mg, 15%), which was identified as cysteine hydrochloride. The solvent was evaporated from the supernatant solution to give a gum. Trituration with acetone gave **4a** (730 mg, 83%) as a white powder: mp 186–189 °C dec; IR (Nujol) 3325, 2750 br, 1720, 1660, 1565 cm^{-1}; NMR (400 MHz; D_2O) δ 2.70 (3 H, s, NCH_3), 3.33 (1 H, dd, J = 15.4 Hz, J = 6.4 Hz) and 3.50 (1 H, dd, J = 15.4 Hz, J = 4.0 Hz) ($CHCH_2S$), 4.24 (1 H, dd, J = 6.4 Hz, J = 4.0 Hz, $CHCH_2$), and 4.75 (5 H, br s, HOD); mass spectrum (LSIMS), m/z 179 [(M + H)$^+$]. Anal. Calcd for $C_3H_{11}ClN_2O_3S$: C, 28.0; H, 5.15; N, 13.05. Found: C, 27.7; H, 5.2; N, 12.75.

***S*-(*N*-Ethylcarbamoyl)cysteine Hydrochloride (4b).** Ester **11b** was hydrolyzed with aqueous HCl, as for the preparation of **4a** above, to give **4b** (81%) as a white powder: mp 177–180 °C dec; optical rotation (c = 354 mM in H_2O) $[\alpha]^{21}_{589}$ −56.2°, $[\alpha]^{21}_{578}$ −58.2°; IR (Nujol) 3300, 2750 br, 1725, 1665 cm^{-1}; NMR (60 MHz; D_2O) δ 1.10 (3 H, t, J = 7 Hz, CH_2CH_3), 3.25 (2 H, q, J = 7 Hz, NCH_2), 3.45 (1 H, d, J = 6 Hz) and 3.50 (1 H, d, J = 4 Hz) ($CHCH_2S$), 4.35 (1 H, dd, J = 6 Hz, J = 4 Hz, $CHCH_2$), 4.9 (5 H, br s, HOD); NMR [300 MHz; $(CD_3)_2SO$] δ 1.04 (3 H, t, J = 7.4 Hz, CH_2CH_3), 3.15 (2 H, dq, J = 6.4 Hz, J = 7.4 Hz, NCH_2), 3.27 (1 H, dd, J = 14.8 Hz, J = 5.6 Hz) and 3.37 (1 H, dd, J = 14.8 Hz, J = 4.6 Hz) ($CHCH_2S$), 4.10 (1 H, ca. t, J = ca. 5 Hz, $CHCH_2$), 8.39 (1 H, ca. t, J = ca. 6 Hz, $NHCH_2$), 8.5 (3 H, br, $CHNH_3^+$); mass spectrum (LSIMS), m/z 193 (100%) [(M + H)$^+$]. Anal. Calcd for $C_6H_{13}ClN_2O_3S$: C, 31.5; H, 5.75; N, 12.25. Found: C, 31.2; H, 5.4; N, 12.0.

***S*-(*N,N*-Dimethylcarbamoyl)cysteine Hydrochloride (4c).** Compound **12** was hydrolyzed with aqueous HCl, as for the preparation of **4a** above, to give **4c** as a white powder: mp 146–150 °C dec; NMR (D_2O) 3.00 [6 H, s, $N(CH_3)_2$], 3.2–3.4 (2 H, m, $CHCH_2S$), 3.50 (1 H, dd, J = 6 Hz, J = 4 Hz, $CHCH_2$), and 4.7 (5 H, br s, HOD); mass spectrum (LSIMS), m/z 193 (100%) [(M + H)$^+$]. Anal. Calcd for $C_6H_{13}ClN_2O_3S$: C, 31.5; H, 5.75; N, 12.25. Found: C, 31.5; H, 5.45; N, 11.95.

***N*-Acetyl-*S*-(*N,N*-dimethylcarbamoyl)cysteine (5).** Compound **4c** (228 mg, 1 mmol) was treated with acetic anhydride (2 mL) and pyridine (100 mg, 1.3 mmol) for 4 h. Evaporation of the excess reagents gave a gum which, after preparative TLC (silica gel; $CHCl_3$/MeOH, 7:1), afforded **5** (140 mg, 60%) as a colorless gum: IR 3300, 2700 br, 1730, 1680, 1660 cm^{-1}; NMR [$(CD_3)_2SO$] δ 2.00 (3 H, s, $COCH_3$), 3.05 [6 H, s, $N(CH_3)_2$], 3.2 (2 H, m, $CHCH_2S$), 4.30 (1 H, m, $CHCH_2$), 7.1 (1 H, br d, J = 6 Hz, NH); mass spectrum, m/z 234 (M^+). Anal. Calcd for $C_8H_{14}N_2O_4S$: C, 41.0; H, 6.0; N, 11.95. Found: C, 40.85; H, 6.25; N, 11.65.

***N*-Acetyl-*S*-(*N*-methylcarbamoyl)cysteine *N*-Methylamide (7).** *N*-Acetylcysteine (**6**) (4.1 g, 25 mmol) was stirred with methyl isocyanate (2.0 g, 35 mmol) in pyridine at 25 °C for 2 days before evaporation of the volatile materials. Trituration with ether gave **7** (4.31 g, 74%) as a white solid: mp 195 °C; optical rotation (c = 45.1 mM in H_2O) $[\alpha]^{21}_{589}$ −23.8°, $[\alpha]^{21}_{578}$ −24.1°, $[\alpha]^{21}_{546}$ −33.3°, $[\alpha]^{21}_{436}$ −53.4°; IR 3300, 3220, 3080, 1675, 1640 cm^{-1}; NMR [$(CD_3)_2SO$] δ 1.90 (3 H, s, $COCH_3$), 2.60 (3 H, d, J = 6 Hz, NCH_3)

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(becomes s on decoupling at δ 7.8), 2.68 (3 H, d, J = 6 Hz, NCH_3) (becomes s on decoupling at δ 7.8), 3.06 (1 H, d, J = 8 Hz) and 3.12 (1 H, s, J = 5 Hz) ($CHCH_2S$), 4.30 (1 H, dt, J = 5 Hz, J = 8 Hz, $CHCH_2$) (becomes d, J = 8 Hz on decoupling at δ 3.1), 7.8 (3 H, br m, NH); mass spectrum, m/z 234 (M^+). Anal. Calcd for $C_8H_{15}N_3O_3S$: C, 41.2; H, 6.5; N, 18.0. Found: C; 41.15; H, 6.25; N, 17.6.

***N*-(*tert*-Butoxycarbonyl)cysteine Methyl Ester (9).** Et_3N (1.01 g, 10 mmol) was added to a well stirred slurry of cysteine methyl ester hydrochloride (8) (1.72 g, 10 mmol) in CH_2Cl_2 (20 mL), followed after 10 min by di-*tert*-butyl dicarbonate (2.18 g, 10 mmol). The mixture was stirred for 16 h, washed with H_2O, and dried. Evaporation of the solvent gave 9 (2.29 g, 97%) as a colorless oil: optical rotation (c = 318 mM in $CHCl_3$) $[\alpha]^{21}_{589}$ +28.5°, $[\alpha]^{21}_{578}$ +29.6°, $[\alpha]^{21}_{546}$ +33.2°, $[\alpha]^{21}_{436}$ +57.6°; IR 3360, 2560, 1745, 1710 cm^{-1}; NMR δ 1.45 [9 H, s, $C(CH_3)_3$], 1.55 (1 H, br, SH), 2.85 (1 H, d, J = 4 Hz) and 3.00 (1 H, d, J = 4 Hz) ($CHCH_2S$), 3.75 (3 H, s, OCH_3), 4.55 (1 H, dt, J = 8 Hz, J = 4 Hz, $CHCH_2$, 5.60 (1 H, d, J = 8 Hz, NH); mass spectrum, m/z 235 (M^+). Anal. Calcd for $C_9H_{17}NO_4S$: C, 45.95; H, 7.3; N, 5.95. Found: C, 46.1; H, 7.3; N, 6.0.

***N*-(*tert*-Butoxycarbonyl)cysteine (10).** Ester 9 (235 mg, 1 mmol) and NaOH (100 mg, 2.5 mmol) were stirred in MeOH (5 mL) under N_2 for 16 h before evaporation of the solvent. Aqueous HCl (1 M; 2.5 mL) was added to the residue to give a solution with final pH = 6.0, which was extracted twice with EtOAc. The combined extracts were washed with saturated aqueous NaCl and were dried. Evaporation of the solvent furnished 10 (150 mg, 68%) as a colorless gum, which was shown to be >97% pure by NMR but would not give a satisfactory microanalysis: IR 3340, 2900–2600, 1710 cm^{-1}; NMR δ 1.40 [9 H, s, $C(CH_3)_3$], 1.50 (1 H, br, SH), 2.9 (2 H, m, $CHCH_2S$), 4.55 (1 H, dt, J = 8 Hz, J = 4 Hz, $CHCH_2$), 5.60 (1 H, d, J = 8 Hz, NH), 8.0 (1 H, br, CO_2H); mass spectrum, m/z 221 (M^+). A small sample was oxidized with I_2 to given *N,N'*-bis(*tert*-butoxycarbonyl)cystine as a white solid: mp 141–143 °C, identical with a commercial sample.

***N*-(*tert*-Butoxycarbonyl)-*S*-(*N*-methylcarbamoyl)cysteine Methyl Ester (11a).** Compound 19 (2.20 g, 9.4 mmol), methyl isocyanate (4 mL), and *N,N*-diisopropylethylamine (0.5 mL) were stirred together in CH_2Cl_2 (20 mL) for 3 days after which time the solvent and excess reagents were evaporated. The residue, in CH_2Cl_2, was washed with H_2O and with saturated aqueous NaCl and was dried. The solvent was evaporated to furnish 11a (2.35 g, 96%) as white needles: mp 45–46 °C; IR 3350, 1735, 1700, 1665 cm^{-1}; NMR δ 1.45 [9 H, s, $C(CH_3)_3$], 2.95 (3 H, d, J = 7 Hz, NCH_3), 3.35 (2 H, d, J = 6 Hz, $CHCH_2S$), 3.70 (3 H, s, OCH_3), 4.45 (1 H, ca. q, J = ca. 6 Hz, $CHCH_2$), 5.85 (1 H, d, J = 7 Hz, OCONH), 6.20 (1 H, br q, J = 7 Hz, MeNH); mass spectrum, m/z 292 (M^+). Anal. Calcd for $C_{11}H_{20}N_2O_5S$: C, 45.15; H, 6.9; N, 9.65. Found: C, 45.45; N, 7.0; N, 9.4.

***N*-(*tert*-Butoxycarbonyl)-*S*-(*N*-ethylcarbamoyl)cysteine Methyl Ester (11b).** Compound 9 was treated with ethyl isocyanate and Et_3N in CH_2Cl_2 as for the preparation of 11a above, to yield 11b (91%) as a colorless oil: IR 3300, 1735, 1700, and 1670 cm^{-1}; NMR δ 1.15 (3 H, t, J = 7 Hz, CH_2CH_3), 1.45 [9 H, s, $C(CH_3)_3$], 3.30 (2 H, quintet, J = 7 Hz, $NHCH_2CH_3$), 3.35 (2 H, d, J = ca. 6 Hz, $CHCH_2S$), 3.70 (3 H, s, OCH_3), 4.45 (1 H, ca. q, J = 6 ca. Hz, $CHCH_2$), 5.55 (1 H, d, J = 7 Hz, OCONH), and 6.00 (1 H, br t, J = 7 Hz, EtNH); mass spectrum, m/z 306 (M^+). Anal. Calcd for $C_{12}H_{22}N_2O_5S$: C, 47.05; H, 7.25; N, 9.15. Found: C, 47.1; H, 7.05; N, 9.0.

***N*-(*tert*-Butoxycarbonyl)-*S*-(*N,N*-dimethylcarbamoyl)cysteine Methyl Ester (12).** Ester 9 (7.05 g, 30 mmol), dimethylcarbamoyl chloride (3.3 g, 30.7 mmol), and pyridine (5.0 g, 63.3 mmol) were stirred together in CH_2Cl_2 (120 mL) for 3 days before the mixture was washed with aqueous H_2SO_4 (1 M) and H_2O. The solution was dried, and the solvent was evaporated to give 12 (2.94 g, 96%) as a colorless oil: optical rotation (c = 251 mM in $CHCl_3$) $[\alpha]^{21}_{589}$ +10.9°; IR 3350, 1740, 1705, 1680 cm^{-1}; NMR 1.40 [9 H, s, $C(CH_3)_3$], 3.05 (3 H, s) and 3.15 (3 H, s) [$CON(CH_3)_2$], 3.1 (2 H, m, $CHCH_2S$), 3.73 (3 H, s, OCH_3), 4.5 (1 H, m, $CHCH_2$), 5.50 (1 H, d, J = 7 Hz, NH); mass spectrum, m/z 306 (M^+). Anal. Calcd for $C_{12}H_{22}N_2O_5S$: C, 47.05; H, 7.25; N, 9.15. Found: C, 47.2; H, 7.1; N, 9.25.

***N*-(Benzyloxycarbonyl)-1,3-thiazolidine-4-carboxylic Acid (14).** Benzyl chloroformate (3.4 g, 20 mmol) was added in one portion to 1,3-thiazolidine-4-carboxylic acid (13) (2.66 g, 20 mmol) and KOH (2.55 g, 45.5 mmol) in H_2O (30 mL), and the whole was stirred vigorously for 16 h. The solution was washed with CH_2Cl_2 and acidified with aqueous HCl (9 M) before being extracted twice with CH_2Cl_2. The combined extracts were dried. The solvent was evaporated to give 14 (4.81 g, 90%); as a colorless gum: NMR δ 3.20 (2 H, d, J = 5 Hz, $CHCH_2S$), 4.45 (1 H, d, J = 9 Hz) and 4.55 (1 H, d, J = 9 Hz) (NCH_2S), 4.85 (1 H, m, $NCHRCO_2H$), 5.20 (2 H, s, OCH_2Ph), 7.30 (5 H, s, ArH), and 9.65 (1 H, s, CO_2H); mass spectrum, m/z 267 (M^+), 221, 91 (100%). A sample was converted to the diisopropylamine salt: NMR δ 1.20 [12 H, d, J = 7 Hz, 2 $CH(CH_3)_2$], 3.17 (2 H, septet, J = 7 Hz, 2 $CH(CH_3)_2$], 3.25 (2 H, m, thiazolidine 5-CH_2), 4.40 (1 H, d, J = 8 Hz) and 4.65 (1 H, d, J = 8 Hz) (thiazolidine 2-CH_2), 4.75 (1 H, m, thiazolidine 4-H), 5.10 (2 H, s, $PhCH_2$), 7.25 (5 H, s, ArH), 8.1 (2 H, br, N^+H_2). Anal. Calcd for $C_{18}H_{25}N_2O_4S$: C, 58.65; H, 7.65; N, 7.6. Found: C, 58.9; H, 7.6; N, 7.65.

***N*-[[*N*-(Benzyloxycarbonyl)-1,3-thiazolidin-4-yl]carbonyl]glycine Benzyl Ester (15).** Acid 14 (1.33 g, 5 mmol) was added to *N,N'*-dicyclohexylcarbodiimide (1.03 g, 5 mmol) in CH_2Cl_2 (20 mL). After 10 min, a mixture of glycine benzyl ester 4-methylbenzenesulfonic acid salt (1.68 g, 5 mmol), *N,N*-diisopropylethylamine (650 mg, 5 mmol), and CH_2Cl_2 (20 mL) was added, and the whole was stirred for 4 days. The evaporation residue was extracted with Et_2O (2 × 50 mL). The combined extracts were washed with H_2O (50 mL), aqueous HCl (2 M; 2 × 50 mL), and saturated aqueous $NaHCO_3$ (2 × 50 mL) before being dried. The filtrate was cooled to 4 °C for 3 days and filtered again. Evaporation of the solvent afforded 15 (1.78 g, 86%) as a colorless oil: IR 3360, 1735, 1680 cm^{-1}; NMR δ 3.30 (1 H, dd, J = 12 Hz, J = 6 Hz) and 3.40 (1 H, dd, J = 12 Hz, J = 4 Hz) (thiazolidine 5-CH_2), 4.00 (2 H, d, J = 5.5 Hz, Gly CH_2), 4.40 (1 H, d, J = 9 Hz) and 4.60 (1 H, d, J = 9 Hz) (thiazolidine 2-CH_2), 4.75 (1 H, dd, J = 6 Hz, J = 4 Hz, thiazolidine 4-H), 5.15 (4 H, s, 2 $PhCH_2O$), 6.7 (1 H, br, NH) and 7.15 (10 H, s, ArH); mass spectrum, m/z 414.1256 (M^+) ($C_{21}H_{22}N_2O_5S$ requires 414.1249), 413, 279, 220, 91 (100%).

***N*-[*N*-(Benzyloxycarbonyl)cysteinyl]glycine Benzyl Ester (16).** Compound 15 (1.0 g, 2.4 mmol) was stirred with $Hg(OAc)_2$ (1.0 g, 3.1 mmol) in AcOH (30 mL) and H_2O (12 mL) at 60 °C for 2 h before being cooled to ambient temperature. A steady stream of H_2S was passed through the solution for 20 min, and the black precipitate of HgS was removed by filtration through diatomaceous earth. Evaporation of the filtrate and AcOH washings gave 16 (903 mg, 94%) as a colorless gum: NMR (400 MHz) δ 1.65 (1 H, dd, J = 7.5 Hz, J = 10.7 Hz, SH), 2.71 (1 H, ddd, J = 6.1 Hz, J = 10.7 Hz, J = 14.0 Hz) and 3.13 (1 H, ddd, J = 4.2 Hz, J = 7.5 Hz, J = 14.0 Hz) (Cys β-CH_2), 4.08 (2 H, d, J = 5 Hz, Gly CH_2), 4.48 (1 H, m, Cys α-H), 5.12 (2 H, s, OCH_2Ph), 5.17 (2 H, s, OCH_2Ph), 5.80 (1 H, br d, J = 7.8 Hz, Cys NH), 6.83 (1 H, br t, J = 5 Hz, Gly NH), 7.35 (10 H, s, ArH); mass spectrum, m/z 402.1246 ($C_{20}H_{22}N_2O_5S$ requires 402.1210), 91 (100%).

***N*-[*N*-(Benzyloxycarbonyl)-*S*-(*N*-methylcarbamoyl)cysteinyl]glycine Benzyl Ester (17).** Methyl isocyanate (2.0 mL) and *N,N*-diisopropylethylamine (1 mL) were added to 16 (470 mg, 1.17 mmol) in CH_2Cl_2 (10 mL), and the whole was stirred for 16 h. The evaporation residue, in CH_2Cl_2, was washed with aqueous HCl (2 M) and H_2O before being dried. Evaporation of the solvent afforded 17 (470 mg, 88%) as a colorless gum: IR 3350, 1725, 1660, 1535 cm^{-1}; NMR (400 MHz) δ 2.83 (3 H, d, J = 4.9 Hz, NCH_3), 3.22 (1 H, dd, J = 14.8 Hz, J = 8.6 Hz) and 3.36 (1 H, dd, J = 14.8 Hz, J = 4.1 Hz) (Cys β-CH_2), 4.05 (1 H, br d, J = 4.7 Hz) and 4.08 (1 H, br d, J = 5.6 Hz) (Gly CH_2), 4.42 (1 H, m, Cys α-H), 5.11 (1 H, d, J = 12.3 Hz) and 5.13 (1 H, d, J = 12.3 Hz) (OCH_2Ph), 5.17 (2 H, s, OCH_2Ph), 5.47 (1 H, br, NH), 6.21 (1 H, br d, J = ca. 6 Hz, Cys-NH), 7.02 (1 H, br, NH), 7.35 (10 H, m, ArH); mass spectrum, m/z 311 [(M − $PhCH_2$ − MeNCO)$^+$], 294, 267, 91 (100%). Anal. Calcd for $C_{22}H_{25}N_3O_6S$: C, 57.5; H, 5.5; N, 9.15. Found: C, 57.2; H, 5.7; N, 8.85.

***N*-[[*N*-(*tert*-Butoxycarbonyl)-1,3-thiazolidin-4-yl]carbonyl]glycine Methyl Ester (19).** Et_3N (520 mg, 5.15 mmol) was added to 18[38] (1.16 g, 5.0 mmol) in CH_2Cl_2 (20 mL), followed after 20 min by isobutyl chloroformate (680 mg, 5.0 mmol), and the mixture was stirred for 1 h. A mixture of glycine methyl ester

hydrochloride (650 mg, 5.2 mmol), Et_3N (540 mg, 5.35 mmol), and CH_2Cl_2 (20 mL) was then added, causing vigorous effervescence. After a further 2 h, the mixture was washed with H_2O, aqueous H_2SO_4 (2 M), aqueous K_2CO_3 (2 M), and H_2O and was dried. The solvent was evaporated to afford **19** (1.46 g, 96%) as a colorless oil: optical rotation (c = 43.6 mM in $CHCl_3$) $[\alpha]^{21}_{589}$ −120.8°, $[\alpha]^{21}_{578}$ −126.9°, $[\alpha]^{21}_{546}$ −145.0°; NMR δ 1.50 [9 H, s, $C(CH_3)_3$], 3.30 (1 H, dd, J = 12 Hz, J = 6 Hz) and 3.40 (1 H, dd, J = 12 Hz, J = 4 Hz) (thiazolidine 5-CH_2), 3.70 (3 H, s, OCH_3), 4.05 (2 H, d, J = 5.5 Hz, Gly CH_2), 4.40 (1 H, d, J = 9 Hz) and 4.65 (1 H, d, J = 9 Hz) (thiazolidine 2-CH_2), 4.70 (1 H, dd, J = 6 Hz, J = 4 Hz, thiazolidine 4-H), 6.9 (1 H, br, NH); mass spectrum, m/z 304 (M^+). Anal. Calcd for $C_{12}H_{20}N_2O_5S$: C, 47.35; H, 6.6; N, 8.95. Found: C, 47.5; H, 6.6; N, 8.95.

***N*-[*N*-(*tert*-Butoxycarbonyl)cysteinyl]glycine Methyl Ester (20).** Compound **29a** (810 mg, 2.13 mmol) was boiled under reflux with propane-1,3-dithiol (1.6 g, 23 mmol) and *N,N*-diisopropylethylamine (700 mg, 5.4 mmol) in anhydrous THF (20 mL) for 2 days. The evaporation residue, in CH_2Cl_2, was washed twice with ice-cold aqueous H_2SO_4 (1 M) and with saturated aqueous NaCl and was dried. Column chromatography of the evaporation residue (silica gel; $CHCl_3$) afforded **20** (590 mg, 95%) as a colorless oil with a distinctive odor: IR 3320, 2560, 1745, 1700, 1660 cm^{-1}; NMR δ 1.45 [9 H, s, $C(CH_3)_3$], 1.72 (1 H, t, J = 8 Hz, SH), 2.7–3.1 (2 H, m, Cys β-CH_2), 3.72 (3 H, s, OCH_3), 4.02 (2 H, d, J = 5 Hz, Gly CH_2), 4.45 (1 H, ddd, J = 8 Hz, J = 7 Hz, J = 5 Hz, Cys α-H), 5.80 (1 H, d, J = 8 Hz, Cys NH), and 7.37 (1 H, t, J = 5 Hz, Gly NH); mass spectrum, m/z 236 [$(M - C_4H_8)^+$] and 220. Anal. Calcd for $C_{11}H_{20}N_2O_5S$: C, 45.2; H, 6.9; N, 9.6. Found: C, 45.5; H, 7.2; N, 9.3.

Experiment to determine the relative lability of *S*-(amidomethyl)- and *N*-(*tert*-butoxycarbonyl) protecting groups. Di-*tert*-butyl dicarbonate (1.09 g, 5 mmol) in Et_2O (10 mL) was added to *S*-[(4-*tert*-butylbenzamido)methyl]glutathione trifluoroacetate salt[18] (**22**) (610 mg, 1 mmol) and Et_3N (1 mL) in H_2O (5 mL), and the whole was stirred vigorously for 3 days. The resulting aqueous solution was washed twice with CH_2Cl_2, and the solvent and excess reagents were removed by freeze-drying to give crude **23** (600 mg) as a gummy white solid: NMR [400 MHz; $(CD_3)_2SO$] δ 1.30 [9 H, s, $ArC(CH_3)_3$], 1.39 [9 H, s, $OC(CH_3)_3$], 2.10 (1 H, m) and 2.18 (1 H, m) (Glu β-CH_2), 2.36 (2 H, m, Glu γ-CH_2), 2.86 (1 H, dd, J = 14.7 Hz, J = 7.3 Hz) and 3.15 (1 H, dd, J = 14.7 Hz, J = 3.6 Hz) (Cys β-CH_2), 3.81 (1 H, d, J = 3.7 Hz) and 3.82 (1 H, d, J = 4.4 Hz) (Gly CH_2), 4.13 (1 H, ca. q, J = ca. 6 Hz, Glu α-CH), 4.59 (1 H, dd, J = 13.5 Hz, J = 6.2 Hz) and 4.66 (1 H, dd, J = 13.5 Hz, J = 6.0 Hz) (NCH_2S), 4.65 (1 H, m, Cys α-CH), 5.61 (1 H, d, J = 6.9 Hz, Glu NH or Cys NH), 7.32 (1 H, ca. t, J = ca. 4 Hz, Gly NH), 7.38 (1 H, d, J = 6.8 Hz, Cys NH or Glu NH), 7.44 (2 H, d, J = 8.4 Hz) and 7.88 (2 H, d, J = 8.4 Hz) (ArH), 8.50 (1 H, ca. t, J = ca. 6 Hz, SCH_2NH). This material (500 mg) was treated with excess CH_2N_2 in Et_2O for 24 h. Careful evaporation of the solvent and excess reagent furnished **24** (500 mg) as a colorless oil of sufficient purity for the next stage: NMR [400 MHz, $(CD_3)_2SO$] δ 1.31 [9 H, s, $ArC(CH_3)_3$], 1.42 [9 H, s, $OC(CH_3)_3$], 2.10–2.20 (2 H, m, Glu β-CH_2), 2.35 (2 H, m, Glu γ-CH_2), 2.85 (1 H, dd, J = 14.5 Hz, J = 7.5 Hz) and 3.15 (1 H, dd, J = 14.5 Hz, J = 3.3 Hz) (Cys β-CH_2), 3.75 (3 H, s, OCH_3), 3.78 (3 H, s, OCH_3), 3.83 (1 H, d, J = 3.8 Hz) and 3.84 (1 H, d, J = 4.3 Hz) (Gly CH_2), 4.15 (1 H, ca. q, J = ca. 6 Hz, Glu α-H), 4.60 (1 H, dd, J = 13.5 Hz, J = 6.2 Hz) and 4.66 (1 H, dd, J = 13.5 Hz, J = 6.0 Hz) (NCH_2S), 4.68 (1 H, m, Cys α-H), 5.73 (1 H, br, NH), 7.31 (1 H, br, NH), 7.39 (1 H, br, NH), 7.45 (2 H, d, J = 8.6 Hz) and 7.91 (2 H, d, J = 8.6 Hz) (ArH), and 8.60 (1 H, ca. t, J = ca. 6 Hz, SCH_2NH). Treatment of a sample (100 mg) of this material with $Hg(OAc)_2$ in 50% aqueous AcOH at 25 °C was without effect during 1 h, but warming to 45 °C for 10 min gave material which had lost the BOC protecting group (primary amine shown by color reaction with indan-1,2,3-trione) whereas no thiol was evident by TLC (comparison with authentic **25**).

***N*-(*tert*-Butoxycarbonyl)glutathione Dimethyl Ester (25).** Compound **36** (800 mg, 1.53 mmol) was boiled under reflux with propane-1,3-dithiol (1.2 g, 8.0 mmol) and *N,N*-diisopropylethylamine (400 mg, 3.1 mmol) in anhydrous THF (10 mL) under N_2 for 2 days. The evaporation residue, in CH_2Cl_2, was washed with ice-cold aqueous H_2SO_4 (2 M) and H_2O and was dried. Chromatography (silica gel; $CHCl_3$/MeOH, 50:1) of the yellow oily evaporation residue gave unreacted disulfide **36** (260 mg, 32%) and **25** (330 mg, 50%) as a white solid: mp 94–95 °C; NMR (400 MHz) δ 1.43 [9 H, s, $OC(CH_3)_3$], 1.82 (1 H, dd, J = 10.0 Hz, J = 7.9 Hz, SH), 1.96 (1 H, ca. dq, J = ca. 14 Hz, J = 7 Hz) and 2.21 (1 H, ca. dq, J = 14 Hz, J = 7 Hz) (Glu β-CH_2), 2.37 (2 H, t, J = 7.2 Hz, Glu γ-CH_2), 2.78 (1 H, m) and 3.13 (1 H, ddd, J = 14.0 Hz, J = 7.9 Hz, J = 4.6 Hz) (Cys β-CH_2), 3.74 (3 H, s, OCH_3), 3.75 (3 H, s, OCH_3), 4.00 (1 H, dd, J = 18.1 Hz, J = 5.4 Hz) and 4.08 (1 H, dd, J = 18.1 Hz, J = 5.8 Hz) (Gly CH_2), 4.34 (1 H, m, Glu α-H), 4.68 (1 H, ddd, J = 8.5 Hz, J = 6.0 Hz, J = 4.6 Hz, Cys α-H), 5.29 (1 H, d, J = 8.5 Hz, Cys NH), 6.86 (1 H, d, J = 7.0 Hz, Glu NH), 6.97 (1 H, ca. t, J = ca. 5.5 Hz, Gly NH); mass spectrum, m/z 435 (M^+), 335, 144, 84 (100%), 57. Anal. Calcd for $C_{17}H_{29}N_3O_8S$: C, 46.9; H, 6.7; N, 9.65. Found: C, 46.6; H, 6.5; N, 9.4.

Attempted Coupling of *N*-(*tert*-Butoxycarbonyl)-*S*-(*tert*-butylthio)cysteine (26a) with Glycine *tert*-Butyl Ester by the Mixed-Anhydride Method. Compound **26a** (309 mg, 1 mmol) was stirred with Et_3N (120 mg, 1.2 mmol) and isobutyl chloroformate (137 mg, 1 mmol) in CH_2Cl_2 (10 mL) for 20 min before addition of a mixture of CH_2Cl_2 (5 mL), Et_3N (110 mg, 1.1 mmol), and glycine *tert*-butyl ester hydrochloride (170 mg, 1 mmol). After a further 15 min, the mixture was washed with H_2O, aqueous H_2SO_4 (2 M), and H_2O and was dried. Chromatography of the evaporation residue (silica gel; $CHCl_3$) yielded **27** (60 mg, 14%) as an unstable colorless gum, which did not give a satisfactory microanalysis but appeared to be >95% pure by NMR; IR 3350, 1700, 1680 cm^{-1}; NMR δ 1.30 [9 H, s, $SC(CH_3)_3$], 1.33 [9 H, s, $SC(CH_3)_3$], 1.47 (9 H, s, $OC(CH_3)_3$], 3.10 (2 H, br d, J = 6 Hz, CH_2S), 4.70 (1 H, ca. q, J = ca. 6 Hz, CH), 5.40 (1 H, br d, J = ca. 7 Hz, NH). From later running fractions was obtained a colorless gum (230 mg), which was shown by NMR to comprise 85 mol % **29b**, identical with the material described below and 15 mol % **28b**: NMR δ 1.45 [18 H, s, 2 $C(CH_3)_3$], 3.95 (2 H, d, J = 5 Hz, Gly CH_2), 5.20 (1 H, m) and 6.00 (1 H, d, J = 1.5 Hz) (propenoyl 3-CH_2), 6.5 (1 H, br, Gly NH), 7.2 (1 H, ca. d, J = ca. 2 Hz, NH).

Attempted Coupling of *N,N'*-Bis(*tert*-butoxycarbonyl)-cystine (26b) with Glycine Methyl Ester by the Mixed-Anhydride Method. Compound **26b** (1.76 g, 4 mmol) was treated with Et_3N (810 mg, 8 mmol) and isobutyl chloroformate (1.09 g, 8 mmol) in CH_2Cl_2 (10 mL) for 20 min before addition of Et_3N (810 mg, 8 mmol) and glycine methyl ester hydrochloride (1.0 g, 8 mmol) in CH_2Cl_2 (20 mL). After a further 45 min, the mixture was washed with H_2O and aqueous H_2SO_4 (2 M) and was dried. Chromatography of the evaporation residue (silica gel; $CHCl_3$) yield *N*-[2-[(*tert*-butoxycarbonyl)amino]propenoyl]glycine methyl ester (**28a**) (490 mg, 24%) as an unstable colorless gum: IR 3300, 1720, 1690 cm^{-1}; NMR (400 MHz) δ 1.46 [9 H, s, $C(CH_3)_3$], 4.10 (2 H, d, J = 6 Hz, Gly CH_2), 5.13 (1 H, t, J = 1.7 Hz becomes d, J = 1.7 Hz on decoupling at δ 7.3) and 6.04 (1 H, d, J = 1.7 Hz) (propenoyl 3-CH_2), 7.3 (1 H, ca. d, J = ca. 2 Hz, NH); mass spectrum, m/z 258.1215 (M^+) ($C_{11}H_{18}N_2O_5$ requires 258.1216), 202, 185, 57 (100%).

***N*-[*N*-(Benzyloxycarbonyl)cysteinyl]glycine Methyl Ester Disulfide (29c).** *N,N'*-Bis(benzyloxycarbonyl)cystine (**26c**) (508 mg, 1 mmol) was treated with isobutyl chloroformate, Et_3N, and glycine methyl ester hydrochloride, as for the reaction of **26b** above, to give a gum comprising mixed methyl and 2-methylprop-1-yl esters of *N*-[*N*-(benzyloxycarbonyl)cysteinyl]glycine disulfide as determined by NMR. This mixture was stirred for 2 days with MeOH (30 mL) and Et_3N (1 mL). Chromatography (silica gel; $CHCl_3$) furnished **29c** (430 mg, 66%) as a white powder: mp 168–171 °C (lit.[40] mp 170–171 °C); NMR δ 3.2 (4 H, m, 2 Cys β-CH_2), 3.70 (6 H, s, 2 OCH_3), 4.00 (4 H, d, J = 6 Hz, 2 Gly CH_2), 4.55 (2 H, m, 2 Cys α-H), 5.10 (4 H, s, 2 $PhCH_2$), 5.8 (4 H, br, 4 NH), 7.30 (10 H, ArH).

***N*-[*N*-(*tert*-Butoxycarbonyl)-*S*-(*tert*-butylthio)cysteinyl]glycine Methyl Ester (29a).** Compound **30** (320 mg, 0.74 mmol) was stirred with glycine methyl ester hydrochloride (251 mg, 2 mmol) and *N,N*-diisopropylethylamine (600 mg, 4.5 mmol) in CH_2Cl_2 (6 mL) for 4 h. The mixture was washed with aqueous NaOH (2 M), H_2O (thrice), aqueous H_2SO_4 (2 M; twice), and H_2O and was dried. Evaporation of the solvent afforded **29a** (270 mg, 96%) as a white solid: mp 105–107 °C; optical rotation (c = 279 mM in $CHCl_3$) $[\alpha]^{21}_{589}$ −56.7°, $[\alpha]^{21}_{578}$ −59.4°, $[\alpha]^{21}_{546}$ −68.0°; IR

3350, 1725, 1685 cm^{-1}; NMR (300 MHz) δ 1.24 [9 H, s, SC(CH$_3$)$_3$], 1.46 [9 H, s, OC(CH$_3$)$_3$], 3.08 (2 H, m, Cys β-CH$_2$), 3.75 (3 H, s, OCH$_3$), 4.03 (1 H, dd, J = 18.3 Hz, J = 5.2 Hz) and 4.08 (1 H, dd, J = 18.3 Hz, J = 5.2 Hz) (Gly CH$_2$), 4.43 (1 H, ca. q, J = ca. 6 Hz, Cys α-CH), 5.29 (1 H, d, J = 7 Hz, Cys NH), 6.82 (1 H, br t, J = ca. 5 Hz, Gly NH); mass spectrum, m/z 381.1512 [(M + H)$^+$] ($C_{15}H_{29}N_2O_5S_2$ requires 381.1518), 380.1431 (M$^+$) ($C_{15}H_{28}N_2O_5S_2$ requires 380.1440), 325, 268, 224, 57 (100%). Anal. Calcd for $C_{15}H_{28}N_2O_5S_2$: C, 47.35; H, 7.4; N, 7.35. Found: C, 47.5; H, 7.7; N, 7.4. A sample (30 mg) was treated with 35% DCl in D$_2$O for 4 days to give a solution of *N*-[*S*-(*tert*-butylthio)cysteinyl]glycine deuteriochloride: NMR (DCl/D$_2$O) δ 1.40 [9 H, s, SC(CH$_3$)$_3$], 3.40 (2 H, d, J = 6 Hz, cys β-CH$_2$), 4.20 (2 H, s, Gly CH$_2$), 4.62 (1 H, t, J = 6 Hz, Cys α-H).

***N*-[*N*-(*tert*-Butoxycarbonyl)-*S*-(*tert*-butylthio)cysteinyl]glycine Methyl Ester (29a) by the Mixed-Anhydride Method.** Compound **26a** was treated with isobutyl chloroformate, Et$_3$N, and glycine methyl ester hydrochloride, as for the reaction of **26b** above, to give, after chromatography, **29a** (44%) as a white solid identical with the material described above.

***N*-[*N*-(*tert*-Butoxycarbonyl)-*S*-(*tert*-butylthio)cysteinyl]glycine *tert*-Butyl Ester (29b).** Ester **30** was treated with glycine *tert*-butyl ester hydrochloride and *N*,*N*-diisopropylethylamine, as for the preparation of **29a** above, to furnish **29b** (240 mg, 95%) as a colorless gum: optical rotation (c = 57.0 mM in CHCl$_3$) $[\alpha]^{21}_{589}$ −11.6°; IR 3320, 1730, 1670 cm^{-1}; NMR δ 1.28 [9 H, s, SC(CH$_3$)$_3$], 1.41 [18 H, s, 2 OC(CH$_3$)$_3$], 3.04 (2 H, ca. d, J = ca. 6 Hz, becomes s on decoupling at δ 4.40, Cys β-CH$_2$), 3.86 (2 H, d, J = 5 Hz, Gly CH$_2$), 4.40 (1 H, ca. q, J = ca. 7 Hz, becomes ca. t, J = ca. 6 Hz on decoupling at δ 5.60, becomes d, J = 7 Hz on decoupling at δ 3.04, Cys α-H), 5.60 (1 H, d, J = 7 Hz, Cys NH), and 6.99 (1 H, ca. t, J = ca. 5 Hz, becomes s on decoupling at δ 3.86, Gly NH). Anal. Calcd for $C_{18}H_{34}N_2O_5S_2$: C, 51.15; H, 8.1; N, 6.65. Found: C, 51.05; H, 8.0; N, 6.5.

***N*-(*tert*-Butoxycarbonyl)-*S*-(*tert*-butylthio)cysteine 4-Nitrophenyl Ester (30).** *N*-(*tert*-Butoxycarbonyl)-*S*-(*tert*-butylthio)cysteine (**26a**) (309 mg, 1 mmol) was stirred with dicyclohexylcarbodiimide (206 mg, 1 mmol) and 4-nitrophenol (139 mg, 1 mmol) in CH$_2$Cl$_2$ (5 mL) for 2 h before being filtered. Chromatography of the evaporation residue (silica gel; CHCl$_3$) afforded **30** (330 mg, 77%) as white needles: mp 102–103 °C; optical rotation (c = 146 mM in CHCl$_3$) $[\alpha]^{21}_{589}$ +25.2°, $[\alpha]^{21}_{578}$ +27.3°, $[\alpha]^{21}_{546}$ +31.1°; IR 1750, 1690, 1510, 1330 cm^{-1}; NMR δ 1.35 [9 H, s, SC(CH$_3$)$_3$], 1.48 [9 H, s, OC(CH$_3$)$_3$], 3.28 (2 H, d, J = 6 Hz, β-CH$_2$), 4.78 (1 H, m, α-CH), 5.65 (1 H, d, J = 8 Hz, NH), 7.30 (2 H, d, J = 9 Hz, Ar 2,6-H), and 8.20 (2 H, d, J = 9 Hz, Ar 3,5-H); mass spectrum, m/z 318 [(M − 2 C$_4$H$_8$)$^+$], 301, 274, 208, 57 (100%). Anal. Calcd for $C_{18}H_{26}N_2O_6S_2$: C, 50.25; H, 6.1; N, 6.5. Found: C, 50.55; H, 6.05; N, 6.25.

***N*-[*N*-(*tert*-Butoxycarbonyl)-*S*-(*N*-methylcarbamoyl)cysteinyl]glycine Methyl Ester (31a).** Compound **20** (270 mg, 0.92 mmol) was stirred with methyl isocyanate (570 mg, 10 mmol) and *N*,*N*-diisopropylethylamine (300 mg, 2.3 mmol) in CH$_2$Cl$_2$ (12 mL) for 3 days before the mixture was washed twice with ice-cold aqueous H$_2$SO$_4$ (1 M), with H$_2$O and with saturated aqueous NaCl. The solution was dried, and the solvent and excess isocyanate were evaporated to furnish **31a** (244 mg, 70%) as an unstable colorless oil, which did not give a satisfactory microanalysis but was shown to be >97% pure by NMR: IR 3350, 1735, 1665 cm^{-1}; NMR δ 1.43 [9 H, s, C(CH$_3$)$_3$], 2.95 (3 H, d, J = 5 Hz, NCH$_3$), 3.2 (2 H, m, Cys β-CH$_2$), 3.72 (3 H, s, OCH$_3$), 4.05 (2 H, d, J = 5 Hz, Gly CH$_2$), 4.35 (1 H, m, Cys α-H), 6.08 (1 H, d, J = 7 Hz, Cys NH), 6.90 (1 H, t, J = 5 Hz, Gly NH), and 7.20 (1 H, q, J = 5 Hz, NHCH$_3$); mass spectrum, m/z 236 [(M − C$_4$H$_8$ − MeNCO)$^+$] and 192.

***N*-[*N*-(*tert*-Butoxycarbonyl)-*S*-(*N*-ethylcarbamoyl)cysteinyl]glycine Methyl Ester (31b).** Compound **20** (270 mg, 0.92 mmol) was treated with ethyl isocyanate (600 mg, 8.5 mmol), as for the preparation of **31a** above, to give **31b** (235 mg, 70%) as an unstable colorless gum: NMR δ 1.14 (3 H, t, J = 7 Hz, CH$_2$CH$_3$), 1.43 [9 H, s, C(CH$_3$)$_3$], 3.1–3.4 (4 H, m, Cys β-CH$_2$ + NCH$_2$CH$_3$), 3.71 (3 H, s, OCH$_3$), 4.02 (2 H, d, J = 5 Hz, Gly CH$_2$), 4.3 (1 H, m, Cys α-H), 5.98 (1 H, d, J = 7 Hz, Cys NH), 6.73 (1 H, t, J = 5 Hz, NHEt or Gly NH), and 7.39 (1 H, t, J = 5 Hz, Gly NH or NHEt); mass spectrum, m/z 236 [(M − C$_4$H$_8$ − EtNCO)$^+$] and 192.

***N*-(*tert*-Butoxycarbonyl)-*S*-(*tert*-butylthio)cysteine *tert*-Butyl Thioester (32).** Ester **30** (430 mg, 1 mmol) was stirred with *N*,*N*-diisopropylethylamine (516 mg, 4 mmol) in 2-methylpropane-2-thiol (5 mL) for 16 h under reflux. Evaporation of excess reagent gave an oil which, in CH$_2$Cl$_2$, was washed with ice-cold aqueous H$_2$SO$_4$ (1 M) and with saturated aqueous NaCl and was dried. Chromatography of the evaporation residue (silica gel; EtOAc/hexane, 1:4) afforded **32** (156 mg, 41%) as a colorless gum, which would not give a satisfactory microanalysis but appeared by NMR and TLC to be >96% pure: NMR δ 1.35 [18 H, s, 2 SC(CH$_3$)$_3$], 1.45 [9 H, s, OC(CH$_3$)$_3$], 3.25 (2 H, d, J = 6 Hz, β-CH$_2$), 4.68 (1 H, m, α-H), and 6.10 (1 H, br, NH).

***N*-(*tert*-Butoxycarbonyl)glutamic Acid α-Methyl γ-(4-Nitrophenyl) Diester (34).** *N*-(*tert*-Butoxycarbonyl)glutamic acid α-methyl ester dicyclohexylammonium salt[31] (**33**) (optical rotation (c = 53.5 m*M* in CHCl$_3$) $[\alpha]^{21}_{589}$ −2.0°) (2.87 g, 6.5 mmol), in CH$_2$Cl$_2$ (80 mL), was washed with aqueous H$_2$SO$_4$ (2 M; 2 × 50 mL) at 0 °C and was dried. The solution was then stirred with dicyclohexylcarbodiimide (1.34 g, 6.5 mmol) and 4-nitrophenol (904 mg, 6.5 mmol) for 24 h before being filtered. Chromatography of the evaporation residue (silica gel; EtOAc/hexane, 1:4) gave **34** (1.93 g, 78%) as a white solid: mp 53–54 °C; IR (Nujol) 3380, 1760, 1735, 1680, 1520, 1350 cm^{-1}; NMR (300 MHz) δ 1.43 [9 H, s, C(CH$_3$)$_3$], 2.01 (1 H, ca. dq, J = 15 Hz, J = 7 Hz) and 2.32 (1 H, ca. dq, J = 15 Hz, J = 7 Hz) (β-CH$_2$), 2.69 (1 H, dt, J = 17.2 Hz, J = 6.8 Hz) and 2.72 (1 H, dt, J = 17.2 Hz, J = 7.5 Hz) (γ-CH$_2$), 3.76 (3 H, s, OCH$_3$), 4.45 (1 H, ca. q, J = ca. 7 Hz, α-H), 5.14 (1 H, d, J = 7.8 Hz, NH), 7.29 (2 H, d, J = 9.2 Hz, Ar 2,6-H), 8.26 (2 H, d, J = 9.2 Hz, Ar 3,5-H); mass spectrum, m/z 382 (M$^+$), 325, 309, 139, 57 (100%). Anal. Calcd for $C_{17}H_{22}N_2O_8$: C, 53.4; H, 5.8; N, 7.35. Found: C, 53.5; H, 5.85; N, 7.4.

***N*-(*tert*-Butoxycarbonyl)-*S*-(*tert*-butylthio)glutathione Bis(methyl ester) (36).** Compound **29a** (1.14 g, 3 mmol) was stirred with CF$_3$CO$_2$H (10 mL) for 16 h before CH$_2$Cl$_2$ (30 mL) was added. This solution was washed with saturated aqueous NaHCO$_3$ and dried. Evaporation of the solvent gave crude **35** (800 mg, 95%) as a colorless oil: NMR δ 1.30 [9 H, s, SC(CH$_3$)$_3$], 2.55 (2 H, br, NH$_2$), 2.70 (1 H, dd, J = 12 Hz, J = 9 Hz) and 3.30 (1 H, dd, J = 12 Hz, J = 3 Hz) (Cys β-CH$_2$), 3.67 (1 H, dd, J = 9 Hz, J = 3 Hz, Cys α-H), 3.70 (3 H, s, OCH$_3$), 4.02 (2 H, d, J = 5 Hz, Gly CH$_2$), and 7.9 (1 H, ca. t, J = ca. 5 Hz, NH). This amine was boiled under reflux with **34** (764 mg, 2 mmol), *N*,*N*-diisopropylethylamine (400 mg, 3.1 mmol), and 4-(dimethylamino)pyridine (20 mg) in anhydrous THF (20 mL) for 30 h. The evaporation residue, in CH$_2$Cl$_2$, was washed with H$_2$O (twice), aqueous H$_2$SO$_4$ (2 M) (twice), 2 M aqueous NaOH (twice), and H$_2$O. The solution was dried, and the solvent was evaporated to afford **36** (878 mg, 84%) as a colorless gum: optical rotation (c = 24.3 mM in CHCl$_3$) $[\alpha]^{21}_{589}$ −47.2°, $[\alpha]^{21}_{578}$ −50.4°, $[\alpha]^{21}_{546}$ −56.7°, $[\alpha]^{21}_{436}$ −103.9°; IR 3320 (br), 1750, 1710–1640 (br) cm^{-1}; NMR (400 MHz) δ 1.32 [9 H, s, SC(CH$_3$)$_3$], 1.42 [9 H, s, OC(CH$_3$)$_3$], 1.93 (1 H, dq, J = 15 Hz, J = 7.5 Hz) and 2.17 (1 H, J = 15 Hz, J = 7.5 Hz) (Glu γ-CH$_2$), 2.36 (2 H, m, Glu β-CH$_2$), 3.09 (1 H, dd, J = 14.0 Hz, J = 6.1 Hz) and 3.13 (1 H, dd, J = 14.0 Hz, J = 6.9 Hz) (Cys CH$_2$), 3.728 (3 H, s, OCH$_3$), 3.734 (3 H, s, OCH$_3$), 4.00 (1 H, dd, J = 18.1 Hz, J = 5.3 Hz) and 4.05 (1 H, dd, J = 18.1 Hz, J = 5.6 Hz) (Gly CH$_2$), 4.37 (1 H, m, Glu α-H), 4.73 (1 H, ca. q, J = ca. 7 Hz, Cys α-H), 5.35 (1 H, d, J = 7.3 Hz, Cys NH or Glu NH), 6.80 (1 H, d, J = 7.2 Hz, Glu NH or Cys NH), and 7.09 (1 H, ca. t, J = ca. 5.5 Hz, Gly NH); mass spectrum, m/z 523 (M$^+$), 467, 450, 434, 367 (100%), 224, 90, 57, 136.72 (M$^+$: 367 → 224). Anal. Calcd for $C_{21}H_{37}N_3O_8S_2$: C, 48.15; H, 7.1; N, 8.0. Found: C, 47.85; H, 7.1; N, 7.7. From one experimental run in the absence of 4-(dimethylamino)pyridine, a gum was obtained which was subjected to column chromatography (silica gel; CHCl$_3$). From the slowest running fraction was isolated 3-[[(*tert*-butylsulfenyl)thio]methyl]-2,5-dioxopiperazine (**38**) (6%) as a white solid: mp 210–214 °C dec; IR (Nujol) 3200, 3050, 1660 cm^{-1}; NMR [CDCl$_3$/(CD$_3$)$_2$SO; 1:1] δ 1.35 [9 H, s, SC(CH$_3$)$_3$], 3.22 (2 H, d, J = 5 Hz, SCH$_2$CH), 3.84 (2 H, m, NHCH$_2$CO), 4.14 (1 H, dt, J = 2 Hz, J = 5 Hz, NHCHCH$_2$) (becomes t, J = 5 Hz on decoupling at δ 8.10), and 8.1 (2 H, br, 2 NH). From other fractions were obtained **36** (59%) and **35** (14%).

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Differences between Rodents and Humans in the Metabolic Toxification of *N,N*-Dimethylformamide

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Differences between Rodents and Humans in the Metabolic Toxification of *N,N*-Dimethylformamide[1]

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Differences between Rodents and Humans in the Metabolic Toxification of *N,N*-Dimethylformamide. MRÁZ, J., CROSS, H., GESCHER, A., THREADGILL, M. D., AND FLEK, J. (1989). *Toxicol. Appl. Pharmacol.* 98, 507–516. The widely used industrial solvent *N,N*-dimethylformamide (DMF) causes liver damage in occupationally exposed persons and is suspected of involvement in the generation of certain occupational malignancies. Here the extent of the biotransformation of DMF to three urinary metabolites has been compared in humans and rodents. The metabolites, which were quantified by gas chromatography (GC) are *N*-(hydroxymethyl)-*N*-methylformamide (HMMF), which yielded *N*-methylformamide on GC analysis, a species which decomposed to formamide on GC analysis, and *N*-acetyl-*S*-(*N*-methylcarbamoyl)cysteine (AMCC), measured after derivatization with ethanol to give ethyl *N*-methylcarbamate. Ten volunteers who absorbed between 28 and 60 μmol/kg DMF during an 8-hr exposure to DMF in the air at 60 mg/m^3 excreted in the urine within 72 hr between 16.1 and 48.7% of the dose as HMMF, between 8.3 and 23.9% as formamide, and between 9.7 and 22.8% as AMCC. AMCC, together with HMMF, was also detected in the urine of workers after occupational exposure to DMF. The portion of the dose (0.1, 0.7, or 7.0 mmol/kg given ip) which was metabolized in mice, rats, or hamsters to HMMF varied between 8.4 and 47.3% of the dose; between 7.9 and 37.5% were excreted as formamide and only between 1.1 and 5.2%, as AMCC. The results suggest that there is a quantitative difference between the metabolic pathway of DMF to AMCC in humans and rodents. It is argued that the hepatotoxic potential of DMF may be linked to the extent of its metabolic conversion to AMCC.

The physical properties of *N,N*-dimethylformamide (DMF)[4] (Fig. 1) make this chemical exceptionally useful as a solvent in the chemical laboratory and in industrial processes, especially for the manufacture of polymers such as polyvinylchloride, polyacrylonitrile, and polyurethanes (Eberling, 1980). World production of DMF is approximately 2×10^5 tons per year (Eberling, 1980). Occupational exposure to DMF causes pancreatic disorders, hepatic damage, and intolerance to alcohol. Recently, carcinomas of the testes have been detected in several individuals in

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[4] Abbreviations used: DMF, *N,N*-dimethylformamide; NMF, *N*-methylformamide; F, formamide; HMMF, *N*-(hydroxymethyl)-*N*-methylformamide; HMF, *N*-(hydroxymethyl)formamide; bisHMF, *N,N*-bis(hydroxymethyl)formamide; AMCC, *N*-acetyl-*S*-(*N*-methylcarbamoyl)cysteine; GC, gas chromatography.

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two populations working with DMF in different industrial settings (Ducatman *et al.*, 1986; Levin *et al.*, 1987). In rodents the toxic potential of DMF seems to be low (for review see Kennedy, 1986; Scailteur and Lauwerys, 1987). There has been one report, as yet uncorroborated, that DMF is carcinogenic in the rat (Kommineni, 1972).

The mechanisms by which DMF causes toxicity are unclear. DMF has been claimed to undergo metabolism to *N*-methylformamide (NMF) (Fig. 1) (Barnes and Ranta, 1972; Kimmerle and Eben, 1975a,b). This biotransformation was thought to be responsible for the liver-damaging properties of DMF (Barnes and Ranta, 1972; Kimmerle and Eben, 1975a) as NMF is a powerful hepatotoxicant in rodents (Whitby *et al.*, 1984; Langdon *et al.*, 1985) and in patients, in whom it has been tested for antineoplastic activity (McVie *et al.*, 1984; Ettinger *et al.*, 1985; Eisenhauer *et al.*, 1986). However, the urinary metabolite of DMF which had been identified as NMF on the basis of gas chromatographic analysis has recently been shown to be *N*-(hydroxymethyl)-*N*-methylformamide (HMMF) (Fig. 1), which is stable in aqueous solution but is thermally degraded to NMF on the gas chromatography column (Brindley *et al.*, 1983; Scailteur and Lauwerys, 1984a; Kestell *et al.*, 1986a). Although the biological properties and metabolic fate of HMMF are presently unknown, studies on its analog *N*-(hydroxymethyl)formamide (HMF) (Fig. 1) (Cooksey *et al.*, 1983) suggest that it is not hepatotoxic.

The hepatotoxicity of monoalkylformamides, such as NMF, in mice appears to be linked to the metabolism of these compounds (Pearson *et al.*, 1987a,b) and, in particular, to a metabolic pathway which leads to the urinary excretion of *N*-acetyl-*S*-(*N*-alkylcarbamoyl)cysteines (AMCC) (Kestell *et al.*, 1987). AMCC (Fig. 1) has been identified as a major metabolite of NMF in patients, rats, and mice (Kestell *et al.*, 1986b; Tulip *et al.*, 1986). Very recently, AMCC has also been identified unequivocally in the urine after exposure of a human to DMF (Mráz and Tureček, 1987). This finding raises the possibility that the mechanisms by which DMF and monoalkylformamides cause hepatic toxicity in humans are related and involve the same reactive intermediate. This paper focuses on the metabolic pathway of DMF which terminates with the excretion of AMCC as it appears to be pivotal for the understanding of the mechanism by which DMF causes toxicity. In particular, the hypothesis has been tested that DMF is biotransformed to AMCC not only in humans (Mráz and Tureček, 1987) but also in rodents. Rodents are most frequently used in investigations of the mechanisms by which solvents such as DMF cause toxicity. Therefore it seems appropriate to establish whether the metabolism of DMF in rodents is qualitatively or quantitatively similar to that observed in humans. To this end, DMF was administered to mice, rats, and hamsters and urine samples were analyzed for the presence of AMCC and other DMF metabolites. The amounts of metabolites found were compared with those determined in the urine of 10 human volunteers after inhalation or ingestion of DMF. Furthermore, in order to determine if AMCC is detectable also under conditions of occupational exposure to DMF, its metabolites were measured in urine samples obtained from 12 workers who were exposed to DMF in their working environment.

METHODS

Chemicals. DMF, NMF, and formamide (F) were purchased from Aldrich Chemical Co. Ltd. (UK). *N*-Acetyl-*S*-(*N*-methylcarbamoyl)cysteine was prepared as described by Kestell *et al.* (1986b). [^{14}C]Methyl-labeled DMF was synthesized by the general method described by Threadgill and Gate (1983).

Animal experiments. DMF was dissolved in saline solution and administered (0.1, 0.7, or 7 mmol/kg) via the ip route to male BALB/c mice (19–26 g), male Sprague-Dawley rats (235–270 g), or male Syrian hamsters (100–114 g). The volume of injection was 0.1 ml in mice and 0.5 ml in rats and hamsters. Animals were housed individually in metabolic cages. Urine samples were col-

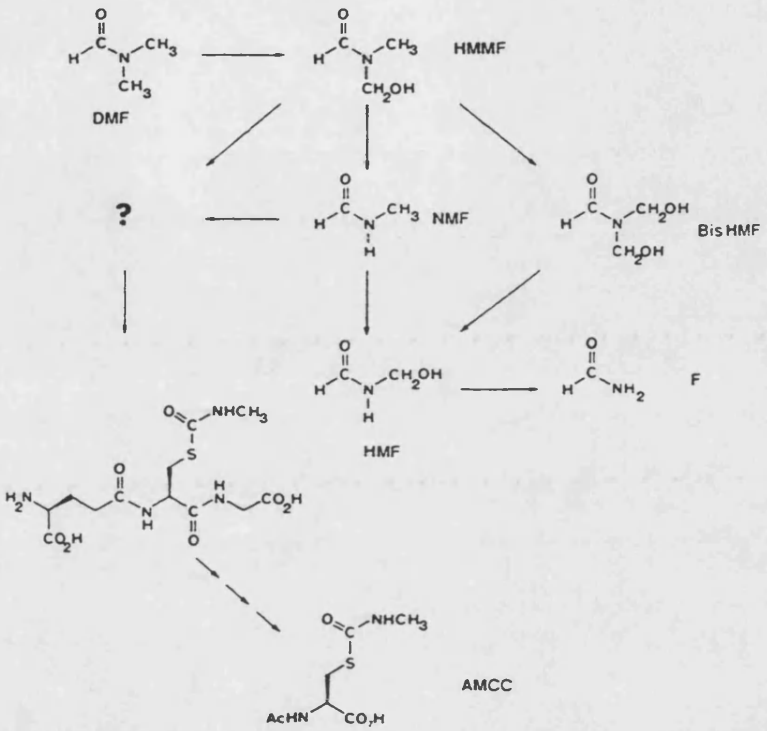

FIG. 1. Proposed metabolic pathway of DMF. So far, of the compounds shown, only HMMF (Kestell *et al.*, 1986a) and AMCC (Mráz and Tureček, 1987) have been unequivocally identified as DMF metabolites. In addition to the metabolites shown, methylamine and dimethylamine are minor DMF metabolites in the mouse (Kestell *et al.*, 1986a).

lected for 72 hr at 12-hr intervals. To ensure quantitative collection of excreted metabolites, cages were rinsed with small volumes of water at each collection.

Exposure of humans. After giving informed consent, 10 healthy volunteers (5 male, 5 female) aged between 26 and 56 years (mean: 41 years) and of 52–94 kg body wt (mean: 70 kg) were exposed for 8 hr to 60 mg/m^3 DMF (20 ppm) in the air. The temperature of the exposure chamber was 25 ± 2°C. The exposure was conducted at the Institute of Hygiene and Epidemiology, Prague. The exposure chamber, designed as described by Ševčivec *et al.* (1974), allowed for the measurement of airborne concentrations of DMF by GC at 5-min intervals and the maintenance of a constant DMF concentration. The dose absorbed *via* inhalation was calculated as the product of DMF vapor concentration, total lung ventilation (measured using a Wright respirometer) during the exposure period, and lung retention of DMF measured by GC analysis of the DMF content in the expired air. DMF absorption via the skin was not taken into account. In a separate study, one of the male volunteers (26 years old) ingested aliquots of DMF (0.02 or 0.1 mmol/kg) dissolved in water (10 ml) at half-hour intervals for 8 hr. Urine was collected at 2- to 8-hr intervals for 4 to 5 days and frozen until analysis by GC.

Early morning urine samples were obtained in the middle of a working week from 12 workers who were occupationally exposed to DMF in a plastics factory in Eastern Bohemia (Czechoslovakia) in which DMF is used to wash polyurethanes off molds. Owing to the preliminary nature of this part of the study, the concentration of DMF vapor at the workplace was not determined.

Analysis of DMF metabolites. Preparation of samples and GC analysis of formamides and AMCC were conducted essentially as described by Mráz *et al.* (1987) and Mráz (1988) using either a Pye Unicam Series 204 or a Chrom 5 (Laboratorní přístroje, Prague) chromatograph, both fitted with nitrogen-sensitive detectors. The method involves derivatization of AMCC with ethanol under alkaline conditions to form ethyl *N*-methylcarbamate prior to GC analysis. The recovery of AMCC as ethyl *N*-methylcarbamate was between 60 and 70% depending on the dilution of the urine sample. Rat urine contained a constituent which cochromatographed with ethyl *N*-

TABLE 1

ACCURACY AND REPRODUCIBILITY OF MEASUREMENT OF AMCC IN URINE SAMPLES FOLLOWING EXTRACTION WITH ETHYL ACETATE[a]

AMCC concentration (μM)	Coefficient of variation (%)[b]		
	Variation on repeated injection	Within-day variation	Between-days variation
20	6.1	8.3	3.8
100	0.9	2.8	6.6

[a] For details of analytical determination see Methods.
[b] Sample size in each case: 6.

methylcarbamate. To eliminate the interference of such endogenous urine constituents and to increase the sensitivity of the method, it was necessary in some cases to extract further and concentrate the ethyl *N*-methylcarbamate. To this end, ethanolic urine extract containing the internal standard was evaporated and the residue was taken up into 1 ml water and extracted into ethyl acetate (1 ml) which was analyzed by GC. The limit of quantitation for AMCC was 1 nmol/ml. The method is characterized by the coefficient of variation values shown in Table 1 for six samples prepared with two concentrations of AMCC. It should be noted that this method determines total *N*-methylcarbamoylating species; previous studies (Mráz and Tureček, 1987; Kestell *et al.*, 1986a) suggest that this is exclusively AMCC in the urine after administration of DMF or NMF.

On the basis of previous work (Brindley *et al.*, 1983; Scailteur and Lauwerys, 1984a; Kestell *et al.*, 1986a), we conclude that the metabolite which was detected as NMF by GC was HMMF which decomposes on the GC column. This metabolite is labeled "NMF" in the following. HMMF itself cannot be detected by HPLC with the required sensitivity. Metabolites of [^{14}C]DMF, of which 0.7 or 7 mmol/kg was given ip, were separated and measured in the urine of six mice by thin-layer chromatography followed by counting of radioactivity as outlined before for metabolites of [^{14}C]NMF (Kestell *et al.*, 1985).

RESULTS

AMCC has never before been described as a metabolite of DMF in rodents. Here we report that AMCC together with "NMF" and DMF were detected and measured in the urine of mice, rats, and hamsters which had received DMF, 0.1, 0.7, or 7 mmol/kg. The amount of DMF excreted as AMCC constitutes between 1.1 and 5.2% of the administered dose (Table 2). Figures 2 to 4 show the time course of excretion of the two DMF metabolites, "NMF" and AMCC. In mice (Fig. 2) these metabolites were excreted rapidly so that they could not be detected beyond 24 hr

TABLE 2

AMOUNTS OF METABOLITES OF DMF IN THE URINE OF MICE, RATS, AND HAMSTERS EXPRESSED AS A PERCENTAGE OF THE DOSE ADMINISTERED IP

Species	DMF dose administered (mmol/kg)	Amount excreted in the urine[a] (% of dose ± SD)			
		DMF	"NMF"	"F"	AMCC
Mouse	7.0	1.2 ± 0.7	45.5 ± 14.3	16.3 ± 7.7	1.1 ± 0.3
	0.7	0.1 ± 0.1	18.2 ± 4.8	27.6 ± 4.9	1.3 ± 0.4
	0.1	0	8.4 ± 3.5	26.0 ± 4.9	1.6 ± 0.4
Rat	7.0	5.5 ± 1.3	44.6 ± 6.1	8.3 ± 2.1	1.7 ± 0.2
	0.7	1.0 ± 0.3	43.2 ± 6.8	15.5 ± 2.8	2.7 ± 0.8
	0.1	0	36.8 ± 2.6	37.5 ± 3.5	5.2 ± 1.0
Hamster	7.0	2.2 ± 0.6	47.3 ± 8.0	7.9 ± 2.7	1.5 ± 0.4
	0.7	0.3 ± 0.1	44.9 ± 18.9	24.0 ± 10.7	2.8 ± 1.0
	0.1	0	29.0 ± 6.9	22.9 ± 4.5	1.9 ± 0.4

[a] Values were obtained in six or seven animals. Measurable amounts of metabolites were detected in mice up to 24 hr, in rats up to 60 hr, and in hamsters up to 36 hr after dosing.

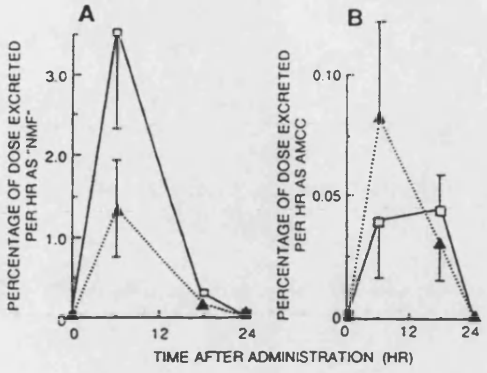

FIG. 2. Time course of excretion of the metabolite which gave NMF on GC analysis and is presumably HMMF (A) and of AMCC (B) in the urine of BALB/c mice after ip administration of 0.7 (▲) or 7 mmol/kg DMF (□). Values are the mean ± SD of six animals.

after drug administration. In rats which received 7 mmol/kg DMF the excretion of the DMF metabolites was delayed. AMCC was not detectable before 24 hr had elapsed after DMF administration (Fig. 3). In hamsters (Fig. 4) "NMF" was excreted rapidly, and the excretion of AMCC after the high dose of DMF was slightly delayed. In accordance with previous reports (Brindley *et al.*, 1983; Scailteur and Lauwerys, 1984a; Kestell *et al.*, 1986a), "NMF," which was most likely HMMF which decomposes to NMF on GC analysis, was found to be the major or one of the major products of the biotransformation of DMF in all three species. A metabolite which gave F on GC analysis and which is labeled "F" in the tables was also a major excretion product. The experiments in mice were repeated using [^{14}C]methyl-labeled DMF. The proportion of the injected radioactivity which was recovered in the urine of six mice was 55 ± 8% after a lc ν (0.7 mmol/kg) and 75 ± 7% after a high dose (7 mmol/kg). The amounts of metabolites measured by counting of radioactivity (results not shown) were very similar to those determined by GC, except in the case of the species which cochromatographed with F. Only 18.3 ± 2.0% of the low and 8.2 ± 1.2% (n = 6) of the high dose produced this metabolite. The exact chemical nature of this product of biotransformation of DMF is currently under investigation and preliminary evidence suggests that it is a mixture containing HMF (see Fig. 1) as the major component. HMF would decompose to F during gas chromatographic analysis.

In order to find out how the amount of metabolites of DMF generated in rodents compares with those formed in humans, samples of urine of 10 volunteers were analyzed after

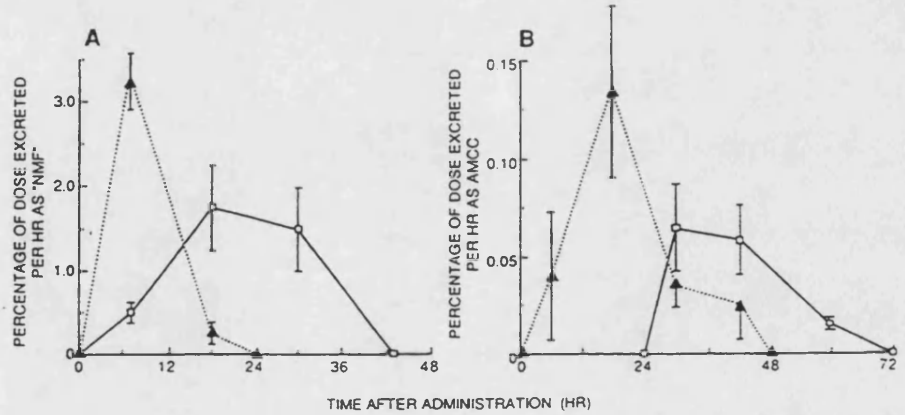

FIG. 3. Time course of excretion of the metabolite which gave NMF in GC analysis (A) and of AMCC (B) in the urine of Sprague–Dawley rats after ip administration of 0.7 (▲) or 7 mmol/kg DMF (□). Values are the mean ± SD of six rats.

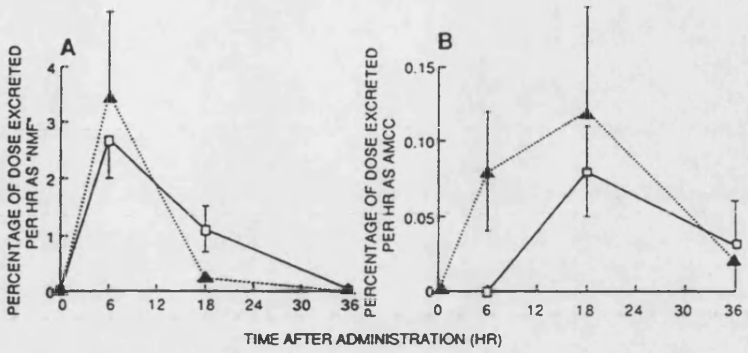

FIG. 4. Time course of excretion of the metabolite which gave NMF on GC analysis (A) and of AMCC (B) in the urine of Syrian hamsters after ip administration of 0.7 (▲) or 7 mmol/kg DMF (□). Values are the mean ± SD of six animals.

exposure to airborne DMF at 60 mg/m^3 which is twice the threshold limit value as defined in a number of countries including the United States. The mean of the amount of DMF absorbed via the lung was 49.3 μmol/kg, which is half of the lowest dose administered to rodents. The results of the analysis (Table 3) show that, in accordance with a previous report (Kimmerle and Eben, 1975b), only a very small proportion of the dose was excreted unchanged. "NMF," "F," and AMCC were three major DMF metabolites excreted by the volunteers. In a separate study, one of the volunteers ingested rather than inhaled DMF, either 0.02 or 0.1 mmol/kg. The time course of excretion of metabolically generated "NMF" and AMCC after oral ingestion of DMF is shown in Fig. 5. "NMF" was detected almost immediately after the start of the period of DMF administration. The volunteer excreted 17.7% of the lower and 17.0% of the higher oral dose as "NMF" compared to 25.6% after exposure to DMF vapor. Most of this metabolite was excreted within 12 hr with peak amounts occurring at the end of the period of ingestion. In contrast, excretion of AMCC did not start until the end of the oral administration and peak concentrations were reached 24 hr later (Fig. 5B). AMCC was detectable in the urine for 5 days and the volunteer excreted 8.5% of the lower and 9.1% of the higher oral dose as AMCC compared to 15.8% after exposure to airborne DMF. The characteristics of the metabolic profile obtained after exposure of the volunteers to DMF vapor (results not shown) were identical to those shown in Fig. 5.

In a preliminary experiment, it was investigated whether AMCC is also detectable in the urine of workers occupationally exposed to DMF. AMCC was indeed discovered in each urine sample obtained from 12 workers who had been exposed regularly to DMF in their working environment. The concentrations varied between 10 and 590 μM (mean: 109 μM). The concentrations of "NMF" found in these urine samples fluctuated between 14 and 112 μM (mean: 45 μM) and the mean ± SD of the ratios of concentrations of AMCC to "NMF" in individual workers was 2.3 ± 1.5. These findings are comparable with those of Maloňová and Bardoděj (1983). They reported an increase in urinary concentrations of constituents which decomposed to thiols on alkaline hydrolysis in workers who had been occupationally exposed to DMF.

DISCUSSION

This is the first time that a quantitative difference has been observed in the metabolism of DMF between humans and rodents. The formation of AMCC is only a minor

TABLE 3

AMOUNTS OF METABOLITES OF DMF IN THE URINE OF 10 VOLUNTEERS EXPRESSED AS A PERCENTAGE OF THE DOSE ABSORBED[a]

	Amount excreted in the urine (% of dose)		
Metabolite	Mean ± SD	Highest value	Lowest value
DMF	0.7 ± 0.4	1.5	0.4
"NMF"	25.9 ± 8.5	48.7	16.1
"F"	14.2 ± 5.2	23.9	8.3
AMCC	14.5 ± 3.6	22.8	9.7

[a] The volunteers were exposed for 8 hr to 60 mg/m^3 DMF in the air and absorbed between 28 and 60 μmol/kg DMF. The mean ± SD of the amount of DMF absorbed was 49.3 ± 11.6 μmol/kg. Metabolites were detected in the urine up to 120 hr after the start of exposure. Further details on the volunteers and measurements are under Methods.

metabolic pathway of DMF in rodents but it is a major route of biotransformation in humans. Whereas in humans 14.5% of the dose absorbed on inhalation of DMF was found as AMCC, the amount measured in mice, rats, or hamsters after ip administration of DMF was only a third or less of this percentage. One could argue that this difference may be partly due to the difference in dose administered. However, this is unlikely because the metabolism of DMF to AMCC in mice or hamsters was not dose-dependent and in rats the percentage of DMF excreted as AMCC increased only threefold on decreasing the dose from 7.0 to 0.1 mmol/kg. It cannot be excluded that the yield of DMF metabolites was to some extent influenced by the route of administration. Indeed, the volunteer who ingested DMF excreted 5% less as AMCC after oral administration than when he inhaled DMF. Absorption of DMF through the skin was not taken into consideration when the dose absorbed on inhalation was determined and therefore the percentages shown in Table 3 may be a slight overestimation. On balance, however, the evidence presented here leaves little doubt that a larger percentage of a dose of DMF is excreted as AMCC in humans than in mice, rats, or hamsters. The results have considerable implications for the assessment of the occupational risk associated with DMF. We have previously demonstrated in mice that there is a mechanistic link between the generation of alkylformamide-induced hepatotoxicity and the urinary excretion of *N*-acetyl-*S*-(*N*-alkylcarbamoyl)cysteines (Kestell *et al.*, 1987). In BALB/c mice in which the hepatotoxic threshold dose of NMF was 3.4 mmol/kg (Kestell *et al.*, 1987), 15% of the dose was excreted as AMCC (Kestell *et al.*, 1985). NMF is also a hepatotoxicant in humans (McVie *et al.*, 1984; Ettinger *et al.*, 1985; Eisenhauer *et al.*, 1986) and undergoes metabolism to AMCC in this species (Kestell *et al.*, 1986b). For example, an oral dose of 14 mmol/m^2 (approximately 0.36 mmol/kg) given three times per week for 4 weeks elicited hepatotoxicity during 11 out of a total of 29 courses of treatment (Eisenhauer *et al.*, 1986). Although AMCC has been identified unequivocally as a metabolite of NMF in the urine of patients (Kestell *et al.*, 1986b), the amount was not measured. In a preliminary experiment, one of the above volunteers ingested NMF (0.1 mmol/kg). Analysis of the urine showed that 17% of the dose was excreted as AMCC (unpublished result), a proportion which is remarkably similar to that produced by mice. In contrast, doses of up to 41 mmol/kg DMF did not cause hepatotoxicity in BALB/c mice (Kestell *et al.*, 1987), and, according to the results presented here, DMF is metabolized to AMCC only to a small extent in this species. Similarly, Sprague–Dawley rats are not very susceptible to the hepatotoxic potential of DMF. A single dose of 14 mmol/kg did not result in liver damage (Scailteur *et al.*, 1981) and, again, the metabolic pathway of DMF leading to the mercapturate was found here to be only of little importance in this species. A single dose of only 6.7 mmol/kg DMF has been claimed to cause the elevation of serum activities of hepatic enzymes in Sprague–Dawley rats. This would suggest damage to the liver (Lundberg *et al.*, 1981). The reason for this dichotomy is

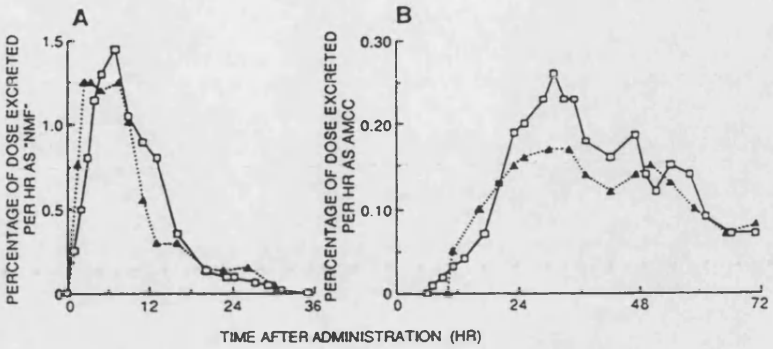

FIG. 5. Time course of excretion of the metabolite which gave NMF on GC analysis (A) and of AMCC (B) in the urine of a volunteer after ingestion of 0.02 (▲) or 0.1 mmol/kg DMF (□).

unclear. If, as seems likely, the metabolic route from DMF to AMCC involves the ultimate hepatotoxic entity as a metabolic intermediate, it is logical to suggest that the relative insensitivity of rodents to DMF-induced hepatotoxicity is related to their poor ability to metabolize DMF via this route. DMF has caused liver damage in exposed workers (Kennedy, 1986; Scailteur and Lauwerys, 1987) and has recently been implicated in the etiology of some occupational testicular cancers (Ducatman *et al.*, 1986; Levin *et al.*, 1987) even though an epidemiological study on 2530 workers did not corroborate an increased cancer incidence linked to DMF exposure (Chen *et al.*, 1988). The findings presented here suggest that the biotransformation of DMF to AMCC is an important pathway in humans but not in rodents. The results cast doubt on the role of rodents as the most appropriate animals to be used in studies designed to help assess the occupational risk associated with exposure to DMF. It remains to be determined whether or not toxic effects of DMF other than hepatic damage are caused by its metabolites. Also, the structures of the ultimate hepatotoxicants formed as metabolites of DMF and of monoalkylformamides are currently unknown.

We have recently postulated that methyl isocyanate (CH_3NCO) or a reactive ester of *N*-methylcarbamic acid (for example, $CH_3NHCOOPO_3^{2-}$) may be toxic metabolites of NMF and precursors of the AMCC found in the urine (Kestell *et al.*, 1987). These reactive species may also be responsible for the toxicity of DMF. Furthermore, thiocarbamates related to AMCC are themselves cytotoxic *in vitro* and *in vivo* (Németh *et al.*, 1978), presumably owing to their carbamoylating properties. Hence, *S*-(*N*-methylcarbamoyl)glutathione or other precursors of AMCC (Threadgill *et al.*, 1987) may cause, or contribute to, the toxicity elicited by DMF or by monoalkylformamides. Currently, the determination of urinary levels of "NMF" is routinely used to assess occupational exposure to DMF. Further studies of the mechanism by which formamides cause toxicity together with the results presented here might suggest that AMCC is a more appropriate biomonitor of exposure to DMF as it is likely to be the product of the metabolic pathway of DMF responsible for toxicity.

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PUBLICATION 35

Structural Studies on Bio-Active Compounds. Part 12. Tautomerism and Conformation of *Ar*-Substituted 1-(2-Hydroxyphenyl)-3-phenylpropane-1,3-diones in the Solid State and in Solution

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Structural Studies on Bio-active Compounds. Part 12.[1] Tautomerism and Conformation of Aryl-substituted 1-(2-Hydroxyphenyl)-3-phenylpropane-1,3-diones in the Solid Phase and in Solution

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The tautomerism of a series of aryl-substituted 1-(2-hydroxyphenyl)-3-phenylpropane-1,3-diones has been studied in deuteriochloroform solution by ^{1}H n.m.r. techniques and, in the case of 1-(2-hydroxy-4-methoxyphenyl)-3-phenylpropane-1,3-dione and 1-(2-hydroxy-4,6-dimethoxyphenyl)-3-phenylpropane-1,3-dione, in the solid state by *X*-ray crystallography. Of these compounds, most exist between 80 and 95% in the enolised form in solution and 1-(2-hydroxy-4-methoxyphenyl)-3-phenylpropane-1,3-dione adopts this tautomer in the crystal. However, 1-(2-hydroxy-4,6-dimethoxyphenyl)-3-phenylpropane-1,3-dione is present as the diketone in the solid phase and enolises very slowly in solution. 1-(6-Benzoyloxy-2-hydroxyphenyl)-3-hydroxy-3-phenylprop-2-en-1-one is shown by ^{1}H n.m.r. spectroscopy possibly to adopt a 'coiled' conformation in solution in deuteriochloroform.

During the preparation of a series of flavones as potential enzyme inhibitors, variously substituted 1-(2-hydroxyphenyl)-3-phenylpropane-1,3-diones (5) were required as intermediates. The enolisation of 1,3-dicarbonyl compounds and the structure of the enol have been the subject of many studies[2,3] by spectroscopic and other methods. Recently, Bassetti *et al.*[4] have inferred from ^{1}H n.m.r. data on simple 1,3-dialkyl- and 1,3-diaryl-propane-1,3-diones that the six-membered hydrogen-bonded ring system in the enol form of these compounds is best described as 'pseudo-aromatic'. It was therefore of interest to study the extent of tautomerism of the 1,3-diphenylpropane-1,3-diones when a potentially hydrogen-bonding *ortho* phenolic hydroxy group is present on one of the phenyl rings and to study the structure of the enols formed.

The synthesis of the required diones was generally straightforward and is shown in Scheme 1. Appropriately substituted 2-hydroxyacetophenones (2) were commercially available or were prepared by a Friedel–Crafts-type acetylation of 3-methoxyphenol (**1a**) or 3,5-dimethoxyphenol (**1b**) using the modification described by Piccolo *et al.*[5] using boron trichloride as a catalyst. In this procedure, an initial complex or borate ester is formed between the phenolic oxygen and the Lewis acid in dichloromethane solution at −10 °C. Addition of acetyl chloride leads to complexation of its carbonyl oxygen to the boron, ensuring that acylation takes place exclusively at the carbon *ortho* to the phenolic hydroxy group. The results presented here show that there is no interference caused by the potentially complexing methoxy group to the regioselectivity of the acylation. This process obviates the need for the harsh conditions of the classical *ortho* acylation of phenol by esterification and subsequent Fries rearrangement at elevated temperature in the presence of aluminium chloride. Phenols (2) were then acylated with a benzoyl chloride (3) in pyridine with or without catalysis by 4-(dimethylamino)pyridine to give the esters (4). Migration of the acyl group from oxygen to carbon was then achieved under base catalysis either by the method of Furniss *et al.*[6] with powdered potassium hydroxide in pyridine or, in the cases of the more labile or recalcitrant esters, under anhydrous and non-nucleophilic conditions using lithium hexamethyldisilazide as the base.

The 400 MHz ^{1}H n.m.r. spectrum of the prototype unsubstituted 1-(2-hydroxyphenyl)-3-phenylpropane-1,3-dione (**5c**) shows the presence of both keto and enol tautomers (Scheme 2) in solution in deuteriochloroform at ambient temperature in the ratio 7:93 respectively. The methylene protons of the keto form resonate as a singlet at δ 4.63; the corresponding vinylic proton of the enol appears as a singlet at δ 6.84. The resonances arising from the OH protons were observed in the downfield portion of the spectrum, as expected for hydroxy groups in strongly hydrogen-bonded environments. Sharp singlets were observed at δ 11.93, 12.19, and 15.94, corresponding to the phenolic OH of the keto and enol tautomers and to the 'enol' respectively. It has been reported by Burdett and Rogers[3] that 1,3-diphenylpropane-1,3-dione is fully enolised in solution in carbon tetrachloride, whereas Bassetti *et al.*[4] observed both keto and enol forms in deuteriochloroform. In the solid state, Weast[7] records the existence of crystals of each tautomer.

The majority of the 1-(2-hydroxyphenyl)-3-phenylpropane-1,3-diones (**5a**, **c–k**) showed greater than 80% enolisation in deuteriochloroform as revealed by their ^{1}H n.m.r. spectra. The dimethoxy compound (**5b**), however, was shown to exist initially as the keto tautomer in solution in this solvent. Progressive enolisation was observed on increasing the temperature from 295 to 325 K in steps of 5 K. This was not reversed on cooling and the final solution contained 38% enol and 62% keto forms of (**5b**). To investigate whether the enolisation of (5) was an effect of dissolution or, alternatively, the enol form was present in the solid state, a determination of the crystal structure of an example of a 1-(2-hydroxyaryl)-3-arylpropane-1,3-dione was carried out by *X*-ray diffraction techniques. Similarly, the solid state structure of the anomalous (**5b**), which is very slowly enolised in solution, was investigated. The monomethoxy analogue (**5a**) was selected as a typical 'enolisable' propane-1,3-dione on two grounds. Firstly, ^{1}H n.m.r. spectroscopy of its deuteriochloroform solution showed it to be *ca.* 92% enolised. Secondly, whereas careful recrystallisation of (**5c–k**) from a variety of solvents furnished only powders, (**5a**) formed large transparent brown crystals from

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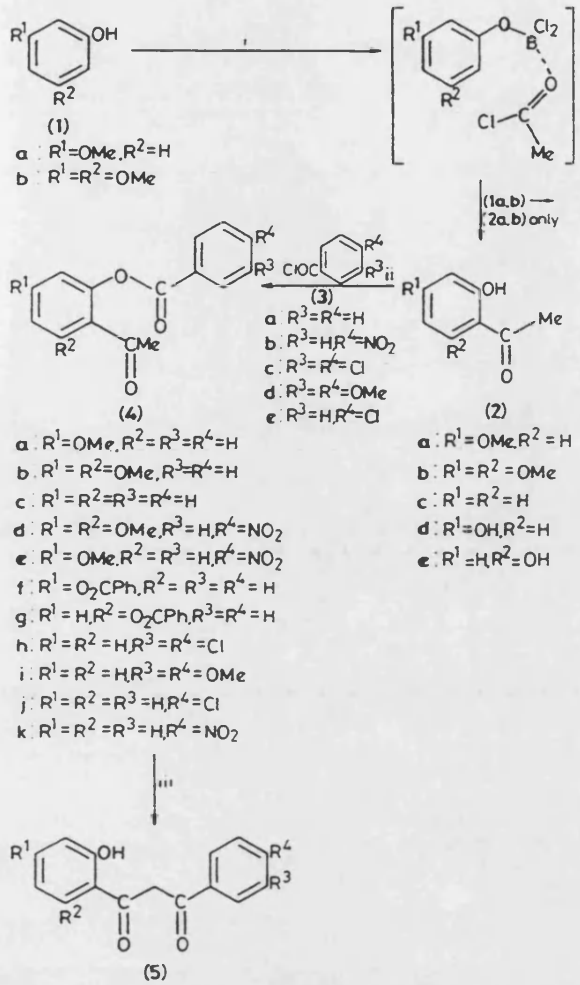
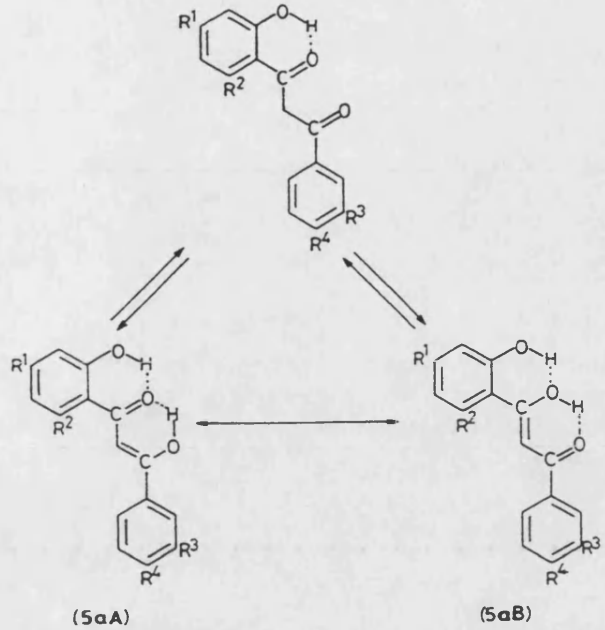


Scheme 1. Synthesis of 1,3-Diarylpropane-1,3-diones (**5**). *Reagents*: i, BCl_3–AcCl–CH_2Cl_2; ii, pyridine +4-(dimethylamino)pyridine; iii, KOH–pyridine or $Li^+ {}^-N(SiMe_3)_2$.



Scheme 2. Keto–enol tautomerism of (**5**) (R^1,R^2,R^3,R^4 as Scheme 1); (**5aA**) and (**5bB**) are limiting structures of the enol of (**5a**) (R^1 = OMe, R^2 = R^3 = R^4 = H).

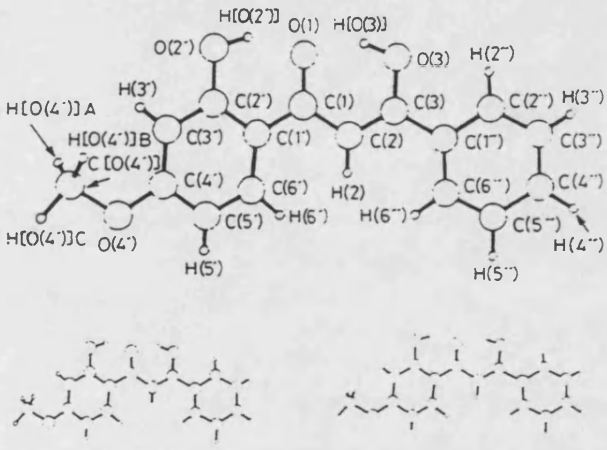


Figure 1. (*a*) Numbering scheme for atoms in (**5a**). (*b*) Stereo plots of (**5a**).

methanol. Similarly, the dimethoxy compound (**5b**) formed elegant hexagonal plates from the same solvent.

Crystal Structure of 1-(2-*Hydroxy*-4-*methoxyphenyl*)-3-*phenylpropane*-1,3-*dione* (**5a**).—The numbering scheme used in the crystallographic determination is shown in Figure 1(*a*).

Crystal data. 1-(2-*Hydroxy*-4-*methoxyphenyl*)-3-*phenylpropane*-1,3-*dione* (**5a**).—$C_{16}H_{14}O_4$, M = 270.3. Monoclinic, a = 10.622(2), b = 12.795(5), c = 10.748(3) Å, β = 111.77(1)°, V = 1 356.6(6) Å^3 (by least-squares refinement on diffractometer angles for 25 automatically centred reflections λ = 0.710 69 Å), space group $P2_1/a$, Z = 4, D_x = 1.321 g cm^{-3}, crystal dimensions = 0.6 × 0.55 × 0.325 mm, μ(Mo-K_α) = 0.57 cm^{-1}.

Crystal Structure of 1-(2-*Hydroxy*-4,6-*dimethoxyphenyl*)-3-*phenylpropane*-1,3-*dione* (**5b**).—The numbering scheme used in the crystallographic determination is shown in Figure 2(*a*).

Crystal data. 1-(4,6-*Dimethoxyphenyl*-2-*hydroxy*)-3-*phenylpropane*-1,3-*dione* (**5b**).—$C_{17}H_{16}O_5$, M = 300.3. Monoclinic, a = 9.503(2), b = 9.924(2), c = 16.125(3) Å, β = 102.22(2)°, V = 1 486.3 Å^3 (by least-squares refinement on diffractometer angles for 25 automatically centred reflections, λ = 0.710 69 Å), space group $P2_1/c$, Z = 4, D_x = 1.439 g cm^{-3}. Crystal dimensions = 0.80 × 0.48 × 0.64 mm, μ(Mo-K_α) = 0.64 cm^{-1}.

Collection and Processing of Data.—Enraf-Nonius CAD4 diffractometer, ω–2θ mode with scan width = 1.0 + 0.35 tan θ, scan speed 0.9–3.3° min^{-1}, graphite monochromated Mo-K_α radiation; for compound (**5a**) 2 671 unique reflections (merging R = 0.093) were measured between 2° ≤ θ < 26° for $+h$, $\pm k$, $\pm l$ giving 1 939 with $|F_o| > 3\sigma(F_o)$; for compound (**5b**), 2 970 unique reflections (merging R = 0.025) were measured between 2° ≤ θ < 26° giving 2 442 with $|F_o| > 3\sigma(F_o)$. No decomposition or movement of the crystal was detected during data collection and refinement. No correction was made for absorption or extinction.

Analysis and Refinement of Structures.—The structure of

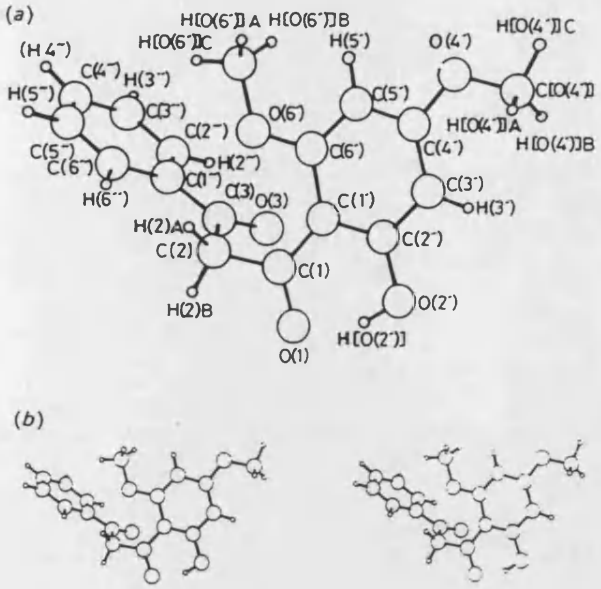


Figure 2. (*a*) Numbering scheme for atoms in (**5b**). (*b*) Stereo plots of (**5b**).

Table 1. Positional parameters (fractional co-ordinates × 10^4) for (**5a**) with estimated standard deviation (esd) in parentheses.

Atom	*x*	*y*	*z*
C(1)	2 766(3)	1 296(2)	3 899(3)
C(2)	2 278(3)	1 960(2)	2 747(3)
C(3)	2 902(3)	2 027(2)	1 862(3)
C(1")	2 424(3)	2 654(2)	621(3)
C(2")	2 981(4)	2 483(3)	−353(3)
C(3")	2 516(4)	3 033(3)	−1 536(4)
C(4")	1 533(4)	3 765(3)	−1 761(4)
C(5")	1 001(4)	3 965(3)	−796(4)
C(6")	1 443(3)	3 411(3)	388(3)
C(1')	2 050(3)	1 199(2)	4 821(3)
C(2')	2 440(3)	445(2)	5 853(3)
C(3')	1 743(4)	340(3)	6 704(3)
C(4')	660(4)	958(3)	6 567(3)
C[O(4')]	311(6)	184(5)	8 437(5)
C(5')	255(4)	1 713(3)	5 573(4)
C(6')	937(4)	1 824(3)	4 727(3)
O(1)	3 831(2)	756(2)	4 114(2)
O(3)	4 048(2)	1 499(2)	2 035(2)
O(2')	3 498(2)	−206(2)	6 049(2)
O(4')	96(2)	912(2)	7 350(2)
H(2")	3 614(28)	1 945(21)	−186(26)
H(3")	2 813(39)	2 858(28)	−2 192(38)
H(4")	1 057(35)	4 129(26)	−2 623(36)
H(5")	212(35)	4 422(27)	950(35)
H(6")	1 065(28)	3 574(21)	1 028(28)
H(2)	1 423(30)	2 261(21)	2 595(26)
H[O(3)]	4 209(35)	1 068(26)	2 803(35)
H(6')	669(46)	2 481(33)	4 045(42)
H(5')	−437(40)	2 081(29)	5 472(39)
H(3')	2 112(32)	−98(24)	7 348(32)
H[O(2')]	3 959(42)	73(32)	5 427(40)
H[O(4')]A	1 250(40)	404(26)	8 983(35)
H[O(4')]B	−501(44)	231(31)	8 849(40)
H[O(4')]C	342(51)	−581(40)	8 067(48)

1-(2-hydroxy-4-methoxyphenyl)-3-phenylpropane-1,3-dione (**5a**) was solved by the use of SHELX.[8] All hydrogen atoms were located by difference electron density synthesis. Final full-matrix least-squares refinement of co-ordinates and anisotropic

Table 2. Bond distances of (**5a**) with esds in parentheses.

Bond	Distance/Å
C(1)–C(2)	1.431(4)
C(1)–C(1')	1.461(4)
C(1)–O(1)	1.272(3)
C(2)–C(3)	1.349(4)
C(3)–C(1")	1.476(4)
C(3)–O(3)	1.343(3)
C(1')–C(2")	1.399(4)
C(1")–C(6")	1.375(4)
C(2")–C(3")	1.374(5)
C(3")–C(4")	1.356(5)
C(4")–C(5")	1.377(5)
C(5")–C(6")	1.378(4)
C(1')–C(2')	1.411(4)
C(1')–C(6')	1.400(4)
C(2')–O(2)	1.351(3)
C(2')–C(3')	1.380(4)
C(3')–C(4')	1.359(4)
C(4')–O(4')	1.362(3)
O(4')–C[O(4')]	1.430(5)
C(4')–C(5')	1.386(5)
C(5')–C(6')	1.364(4)
C(2)–H(2)	0.94(3)
C(2")–H(2")	0.93(3)
C(3")–H(3")	0.90(4)
C(4")–H(4")	0.99(4)
C(5")–H(5")	0.98(3)
C(6")–H(6")	0.94(3)
C(3')–H(3')	0.86(3)
C[O(4')]–H[O(4')]A	0.99(4)
C[O(4')]–H[O(4')]B	1.11(4)
C[O(4')]–H[O(4')]C	1.06(5)
C(5')–H(5')	0.85(4)
C(6')–H(6')	1.08(4)
O(3)–H[O(3)]	0.95(4)
O(2')–H[O(2')]	1.03(4)

Table 3. Interatomic angles for (**5a**) with esds in parentheses.

Atoms	Bond angle/°
C(1')–C(1)–C(2)	121.7(3)
O(1)–C(1)–C(2)	119.5(2)
O(1)–C(1)–C(1')	118.8(2)
C(3)–C(2)–C(1')	122.2(3)
C(1")–C(3)–C(2)	125.3(3)
O(3)–C(3)–C(2)	121.8(3)
O(3)–C(3)–C(1")	112.9(2)
C(2")–C(1")–C(3)	119.5(3)
C(6")–C(1")–C(3)	122.1(2)
C(6")–C(1")–C(2")	118.4(3)
C(3")–C(2")–C(1")	120.4(3)
C(4")–C(3")–C(2")	120.5(3)
C(5")–C(4")–C(3")	119.9(3)
C(6")–C(5")–C(4")	120.3(4)
C(5")–C(6")–C(1")	120.4(3)
C(2')–C(1')–C(1)	121.1(3)
C(6')–C(1')–C(1)	122.7(3)
C(6')–C(1')–C(2')	116.2(2)
C(3')–C(2')–C(1')	120.9(3)
O(2')–C(2')–C(1')	121.9(2)
O(2')–C(2')–C(3')	117.2(3)
C(4')–C(3')–C(2')	120.9(3)
C(5')–C(4')–C(3')	119.8(3)
O(4')–C(4')–C(3')	125.3(3)
O(4')–C(4')–C(5')	114.9(3)
C(6')–C(5')–C(4')	119.8(3)
C(5')–C(6')–C(1')	122.4(3)
C[O(4')]–O(4')–C(4')	117.7(3)

Table 4. Selected torsion angles for (5a).

Atoms	Angle/°
C(1′)–C(1)–C(2)–C(3)	−177.5
O(1)–C(1)–C(2)–C(3)	0.8
C(2)–C(1)–C(1′)–C(2′)	171.8
C(2)–C(1)–C(1′)–C(6′)	−7.1
O(1)–C(1)–C(1′)–C(2′)	−6.5
O(1)–C(1)–C(1′)–C(6′)	174.6
C(1)–C(2)–C(3)–C(1″)	176.9
C(1)–C(2)–C(3)–O(3)	−2.2
C(2)–C(3)–C(1″)–C(2″)	−165.9
C(2)–C(3)–C(1″)–C(6″)	14.4
O(3)–C(3)–C(1″)–C(2″)	13.4
O(3)–C(3)–C(1″)–C(6″)	−166.4
C(3)–C(1″)–C(2″)–C(3″)	177.7
C(6″)–C(1″)–C(2″)–C(3″)	−2.5
C(3)–C(1″)–C(6″)–C(5″)	−178.5
C(2″)–C(1″)–C(6″)–C(5″)	1.7
C(1″)–C(2″)–C(3″)–C(4″)	1.5
C(2″)–C(3″)–C(4″)–C(5″)	0.4
C(3″)–C(4″)–C(5″)–C(6″)	−1.2
C(4″)–C(5″)–C(6″)–C(1″)	0.1
C(1)–C(1′)–C(2′)–C(3′)	−178.6
C(1)–C(1′)–C(2′)–O(2′)	1.1
C(6′)–C(1′)–C(2′)–C(3′)	0.4
C(6′)–C(1′)–C(2′)–O(2′)	−180.0
C(1)–C(1′)–C(6′)–C(5′)	178.5
C(2′)–C(1′)–C(6′)–C(5′)	−0.4
O(2′)–C(2′)–C(3′)–C(4′)	−179.4
C(2′)–C(3′)–C(4′)–C(5′)	−0.8
C(2′)–C(3′)–C(4′)–O(4′)	179.7
C(3′)–C(4′)–C(5′)–C(6′)	0.8
O(4′)–C(4′)–C(5′)–C(6′)	−179.7
C(3′)–C(4′)–O(4′)–C[O(4′)]	2.5
C(5′)–C(4′)–O(4′)–C[O(4′)]	−176.9
C(4′)–C(5′)–C(6′)–C(1′)	−0.1

thermal parameters for non-hydrogen atoms, and co-ordinates and isotropic temperature factors for hydrogen atoms reduced $R = \Sigma\|F_o\| - \|Fc\|/\Sigma|F_o|$ and $R_w = \Sigma w^{\frac{1}{2}}\|F_o\| - \|Fc\|/\Sigma|w^{\frac{1}{2}}|F_o|$ to 0.056 and 0.057 respectively. In the final stages of refinement, reflections were weighted according to $W = k/\sigma^2(F_o)$ where k converged at 2.3867 and $\sigma(F_o)$ was obtained from counting statistics and an allowance for the instability of the instrument. Refinement was terminated when no positional parameter shifted by more than 0.006 esd, at which point a difference electron density map showed no feature greater than ± 0.19 e Å^{-3}.

The structure of 1-(2-hydroxy-4,6-dimethoxyphenyl)-3-phenylpropane-1,3-dione (5b) was solved by the use of the MULTAN78 direct methods package[9] and subsequently refined using SHELX.[8] Again, all hydrogen atoms were located by difference electron density synthesis. Final full-matrix least-squares refinement, as for (5a), reduced R and R_w to 0.042 and 0.041 respectively and reflections in the final stages were weighted according to $w = k/\sigma^2(F_o)$ where k converged at 3.195. The refinement was terminated when no positional parameter shifted by more than 0.002 esd, at which point a difference electron density map showed no feature greater than ± 0.27 e Å^{-3}.

Positional parameters for the structure of (5a) are shown in Table 1, with bond lengths in Table 2, bond angles in Table 3,

* *Supplementary data*: (see section 5.6.3 of Instructions for Authors, in the January issue). Full lists of bond lengths and angles and of thermal parameters have been deposited at the Cambridge Crystallographic Data Centre.

Table 5. Positional parameters (fractional co-ordinates × 10^4) of (5b) with esds in parentheses.

Atom	*x*	*y*	*z*
C(6′)	5 031(2)	2 921(1)	5 792(1)
C(5′)	4 117(2)	2 418(2)	6 266(1)
C(4′)	3 137(2)	1 411(1)	5 934(1)
C(3)′	3 047(2)	912(2)	5 127(1)
C(2′)	3 978(2)	1 424(1)	4 649(1)
C(1′)	5 003(2)	2 442(1)	4 953(1)
C(1)	5 964(2)	2 902(2)	4 420(1)
C(2)	7 085(2)	3 977(2)	4 690(1)
C(3)	8 313(2)	3 508(2)	5 386(1)
C(1″)	9 195(2)	4 514(1)	5 957(1)
C(2″)	10 356(2)	4 064(2)	6 566(1)
C(3″)	11 148(2)	4 966(2)	7 135(1)
C(4″)	10 789(2)	6 307(2)	7 100(1)
C(5″)	9 657(2)	6 763(2)	6 498(1)
C(6″)	8 870(2)	5 884(2)	5 922(1)
O(6′)	5 984(1)	3 923(1)	6 075(1)
C[O(6′)]	6 081(3)	4 428(2)	6 917(1)
O(4′)	2 306(1)	996(1)	6 472(1)
C[O(4′)]	1 224(2)	5(2)	6 185(1)
O(2′)	3 587(1)	902(1)	3 866(1)
O(1)	5 878(1)	2 414(1)	3 697(1)
O(3)	8 587(1)	2 314(1)	5 461(1)
H(5′)	4 161(17)	2 728(16)	6 810(11)
H(3′)	2 402(19)	237(17)	4 880(11)
H[O(6′)]A	5 152(24)	4 790(19)	6 973(13)
H[O(6′)]B	6 400(21)	3 667(19)	7 332(12)
H[O(6′)]C	6 808(20)	5 101(18)	6 987(11)
H[O(4′)]A	478(23)	382(20)	5 747(14)
H[O(4′)]B	1 646(22)	−802(21)	6 040(13)
H[O(4′)]C	771(25)	−139(21)	6 713(16)
H(2)A	6 641(17)	4 785(16)	4 844(10)
H(2)B	7 487(18)	4 193(16)	4 207(11)
H[O(2′)]	4 545(27)	1 358(23)	3 659(14)
H(2″)	10 587(20)	3 118(18)	6 591(11)
H(3″)	11 898(22)	4 629(18)	7 530(13)
H(4″)	11 323(22)	6 922(20)	7 536(14)
H(5″)	9 385(22)	7 736(21)	6 470(12)
H(6″)	8 095(20)	6 228(18)	5 474(11)

and selected torsion angles in Table 4. The corresponding data for the dimethoxy analogue (5b) are reported in Tables 5–8 respectively.*

The crystal structures confirm that (5a) exists in the enol form in the solid state and that (5b) exists in the keto form. As expected, the enol system in (5a) is planar. However, unlike the situation for enols of simple 1,3-diarylpropane-1,3-diones,[3] this six-membered hydrogen-bonded ring is not symmetrical in terms of bond lengths and thus the enol can be described as tending towards either structure (5aA) or structure (5aB) (Scheme 2). The distinction between these two limiting structures is revealed by the differences between the lengths of the two carbon–oxygen bonds, with C(1)–O(1) being 1.273 Å and C(3)–O(3) being 1.343 Å. The former is slightly longer than expected for a ketonic carbonyl group;[10] this may be attributable to a reduction in bond order through hydrogen-bonding both from the phenolic and the enolic OH. The length of C(3)–O(3) is comparable to that of a shortened C–O single bond (*e.g.* in a carboxylic acid) (*ca.* 1.36 Å).[10] That the solid state structure of the enol of (5a) tends to the limit (5aA) is confirmed by comparison of the lengths of C(1)–C(2) (1.431 Å) and C(2)–C(3) (1.349 Å), indicating *approach* to single and double bond character respectively. Indeed, the phenyl–carbonyl bond [C(1′)–C(1)] also has bond order slightly higher than unity with length 1.461 Å compared with the normal alkane C–C bond length of *ca.* 1.541 Å.[10] The shortening of C(1′)–C(1) and C(1)–C(2) reflects

Table 6. Bond distances of (**5b**) with esds in parentheses.

Atoms	Distances
C(5′)–C(6′)	1.368(2)
C(1′)–C(6′)	1.429(2)
C(6′)–O(6′)	1.358(2)
C(4′)–C(5′)	1.393(2)
C(3′)–C(4′)	1.378(2)
O(3′)–C(3′)	1.355(2)
C(2′)–C(3′)	1.387(2)
C(1′)–C(2′)	1.417(2)
C(2′)–O(2′)	1.348(2)
C(1′)–C(1)	1.453(2)
C(1)–C(2)	1.506(2)
C(1)–O(1)	1.249(2)
C(2)–C(3)	1.510(2)
C(3)–C(1″)	1.490(2)
C(3)–O(3)	1.213(2)
C(1″)–C(2″)	1.386(2)
C(1″)–C(6″)	1.393(2)
C(2″)–C(3″)	1.386(2)
C(3″)–C(4″)	1.373(3)
C(4″)–C(5″)	1.365(3)
C(5″)–C(6″)	1.374(3)
C[O(6′)]–O(6′)	1.432(2)
C[O(4′)]–O(4′)	1.427(2)
C(5′)–H(5′)	0.92(2)
C(3′)–H(3′)	0.94(2)
C(2)–H(2)A	0.90(2)
C(2)–H(2)B	0.96(2)
C(2″)–H(2″)	0.96(2)
C(3″)–H(3″)	0.91(2)
C(4″)–H(4″)	0.99(2)
C(5″)–H(5″)	1.00(2)
C(6″)–H(6″)	0.98(2)
C[O(6′)]–H[O(6′)]A	0.98(2)
C[O(6′)]–H[O(6′)]B	1.00(2)
C[O(6′)]–H[O(6′)]C	0.95(2)
C[O(4′)]–H[O(4′)]A	0.96(2)
C[O(4′)]–H[O(4′)]B	0.95(2)
C[O(4′)]–H[O(4′)]C	1.04(3)
O(2′)–H[O(2′)]	0.92(3)

Table 7. Interatomic angles for (**5b**) with esds in parentheses.

Atoms	Bond angle/°
C(1″)–C(6′)–C(5′)	121.5(1)
O(6′)–C(6′)–C(5′)	122.4(1)
O(6′)–C(6′)–C(1′)	116.1(1)
C(4′)–C(5′)–C(6′)	119.9(1)
C(4′)–C(3′)–C(2′)	121.6(1)
O(4′)–C(4′)–C(5′)	113.9(1)
O(4′)–C(4′)–C(3′)	124.5(1)
C(2′)–C(3′)–C(4′)	118.3(2)
C(1′)–C(2′)–C(3′)	122.9(1)
O(2′)–C(2′)–C(3′)	116.2(1)
O(1′)–C(2′)–C(1′)	120.9(1)
C(2′)–C(1′)–C(6′)	115.9(1)
C(1)–C(1′)–C(6)	124.6(1)
C(1)–C(1′)–C(2)	119.5(1)
C(2)–C(1)–C(1′)	123.1(1)
O(1)–C(1)–C(1′)	120.3(1)
O(1)–C(1)–C(2)	116.6(1)
O(3)–C(2)–C(1)	112.1(1)
C(1″)–C(3)–C(2)	119.9(1)
O(3)–C(3)–C(2)	119.4(1)
O(3)–C(3)–C(1″)	120.8(1)
C(2″)–C(1″)–C(3)	118.8(1)
C(6″)–C(1″)–C(3)	122.6(1)
C(6″)–C(1″)–C(2″)	118.6(2)
C(3″)–C(2″)–C(1″)	120.1(2)
C(4″)–C(3″)–C(2″)	120.2(2)
C(5″)–C(4″)–C(3″)	120.2(2)
C(6″)–C(5″)–C(4″)	120.2(2)
C(5″)–C(6″)–C(1″)	120.6(2)
C[O(6′)]–O(6′)–C(6′)	118.7(1)
C[O(4′)]–O(4′)–C(4′)	118.6(1)

the extensive conjugation in this system resulting in co-planarity of the phenyl ring bearing the OH substituent and the enol system. It is interesting to note, however, that the phenyl ring attached to C(3) is not co-planar with the enol system but is at an angle of 14°. While it might initially be thought that there would be further conjugation of the π-bonds of the enol system with the π system of the phenyl ring, consideration of the interatomic distance between the vinyl proton and the aromatic proton *ortho* to the enol system explains this effect. Even at an angle of 14°, the contact distance between H(2) (the vinyl proton) and H(6″) (one of the *ortho* protons) is 2.308 Å. If the phenyl ring were co-planar with the enol system, then these protons would virtually be in contact.

In contrast to the enol system seen in (**5a**), the crystal structure of (**5b**) shows no such tautomerism, although the carbonyl oxygen [O(1)] is hydrogen-bonded to the *ortho* phenolic OH. The C–C single bond lengths of the propane-1,3-dione system are similar to the single bonds in (**5a**), although they are not identical (Tables 2 and 6).

The ^{1}H n.m.r. spectrum of (**5b**) showed a marked difference in chemical shift of the two *O*-methyl groups. This Δδ is surprising when compared with the corresponding value for the precursor benzoate ester (**4b**) (Table 9). It appears that one of the *O*-methyl groups is being unusually shielded. Consideration of the crystal structure and data supplies a reasonable explanation for this effect. Since this compound exists as the diketone, the molecule is not constrained to planarity about the propane-1,3-dione system. In the crystal, the *O*-methyl group in the 6′-position lies very close to the phenyl ring attached to C(3) of the propane-1,3-dione. If this structure were maintained in solution, there would be a contact distance of *ca.* 2.97 Å between one of the protons of the *O*-methyl group [HO(6′)] and C(6″), placing this methyl group within the anisotropic shielding region of the phenyl ring. The shielding is also notable in the ^{1}H n.m.r. spectrum of the 4″-nitro analogue (**5d**) (keto form) with resonances for the methoxy groups at δ 3.53 and 3.82.

A trend is notable in the chemical shifts of the phenolic OH in the enol forms of (**5a**, **c**–**k**) as shown in Table 10. The enols of compounds (**5c**, **h**–**k**) with no substituent in the phenolic ring give $\delta_{\text{Phenol-OH}}$ in the range 11.92–12.10, whereas (**5a**, **e**, **f**) with one electron-donating oxygen substituent give resonances for this proton in the range δ 12.36–12.50 and the dimethoxy compound (**5d**) shows δ_{OH} 13.33. The chemical shift of the enol OH proton varied from δ 15.20 to 15.85 with 4″-electron-withdrawing substituents causing the upfield shift and *vice versa*. Introduction of a 4′-methoxy substituent also resulted in a slight upfield shift of this proton. In contrast, the reported [4] chemical shift for this proton in 3-hydroxy-1,3-diphenylprop-2-en-1-one is δ 16.80. Little variation is seen in the chemical shifts of the vinyl proton in the enol forms of (**5a**, **c**, **e**, **f**, **h**–**k**) in that they lie in the range δ 6.62–6.90, consistent with the corresponding value (δ 6.82) [3] for 3-hydroxy-1,3-diphenylprop-2-en-1-one. The exceptions are (**5b**, **d**), with $\delta_{\text{Vinyl-H}}$ 7.32 and 7.41, respectively, resulting from the electronic or steric effects of the introduction of the 6′-methoxy function.

In an attempt to prepare 5-hydroxyflavone, 2-acetylbenzene-1,3-diol (**2d**) was benzoylated to give the diester (**4g**) which was rearranged to furnish the propane-1,3-dione (**5g**). No hydrolysis of the remaining benzoate ester was evident under the conditions of the rearrangement (hot potassium hydroxide–pyridine). The chemical shifts of the ArH protons of the enol

Table 8. Selected torsion angles for (5b).

Atoms	Angle/°
C(1′)–C(6′)–C(5′)–C(4′)	0.4
C(6′)–C(6′)–C(5′)–C(4′)	178.4
C(2′)–C(1′)–C(6′)–C(5′)	0.0
C(1)–C(1′)–C(6′)–C(5′)	−178.3
O(6′)–C(6′)–C(1′)–C(2′)	−178.2
O(6′)–C(6′)–C(1′)–C(1)	3.5
C(5′)–C(6′)–C(6′)–C[O(6′)]	3.0
C(1′)–C(6′)–O(6′)–C[O(6′)]	−178.9
C(3′)–C(4′)–C(5′)–C(6′)	−0.9
C(6′)–C(5′)–C(4′)–O(4′)	179.5
C(2′)–C(3′)–C(4′)–C(5′)	1.0
C(2′)–C(3′)–C(4′)–O(4′)	−179.4
C(5′)–C(4′)–C(4′)–C[O(4′)]	177.6
C(3′)–C(4′)–O(4′)–C[O(4′)]	−2.1
C(4′)–C(3′)–C(2′)–C(1′)	−0.6
C(4′)–C(3′)–C(2′)–O(2′)	179.7
C(6′)–C(1′)–C(2′)–C(3′)	0.2
C(1)–C(1′)–C(2′)–C(3′)	178.5
O(2′)–C(2′)–C(1′)–C(1)	−179.9
C(2′)–C(1′)–C(1)–O(1)	0.8
C(1′)–C(1)–C(2)–C(3)	70.1
O(1)–C(1)–C(2)–C(3)	−110.5
O(2)–C(2)–C(3)–C(1″)	−156.4
C(1)–C(2)–C(3)–O(3)	25.3
C(2)–C(3)–C(1″)–C(2″)	−177.1
C(2)–C(3)–C(1″)–C(6″)	5.1
O(3)–C(3)–C(1″)–C(2″)	1.2
O(3)–C(3)–C(1″)–C(6″)	−176.5
C(3)–C(1″)–C(2″)–C(3″)	−176.5
C(6″)–C(1″)–C(2″)–C(3″)	1.3
C(3)–C(1″)–C(6″)–C(5″)	175.6
C(2″)–C(1″)–C(6″)–C(5″)	−2.1
C(1″)–C(2″)–C(3″)–C(4″)	0.0
C(2″)–C(3″)–C(4″)–C(5″)	−0.6
C(3″)–C(4″)–C(5″)–C(6″)	0.1
C(4″)–C(5″)–C(6″)–C(1″)	1.5

Table 9. Chemical shifts of methoxy groups in compounds (4b), (5b), and (5d).

Compound	δ_H of OMe groups	
(4b)	3.73	3.75
(5b)	3.43	3.79
(5d) (keto)	3.53	3.82

Table 10. Chemical shifts of phenol OH, vinyl H, and enol OH of enol forms of compounds (5a, c–k).

Compound	Substituents	$\delta_{phenol\ OH}$	$\delta_{vinyl\ H}$	$\delta_{enol\ OH}$
(5a)	4′-OMe	12.50	6.62	15.40
(5c)	unsubstituted	12.10	6.84	15.54
(5d)	4′,6′-$(OMe)_2$, 4″-NO_2	13.33	7.41	15.26
(5e)	4′-OMe, 4″-NO_2	12.44	6.78	15.20
(5f)	4′-O_2CPh	12.36	6.80	15.45
(5g)	6′-O_2CPh	11.91	6.87	15.73
(5h)	3″,4″-Cl_2	11.95	6.78	15.43
(5i)	3″,4″-$(OMe)_2$	12.09	6.75	15.85
(5j)	4″-Cl	11.95	6.70	15.5
(5k)	4″NO_2	11.92	6.90	15.30

form of (5g) were anomalous in the ^1H n.m.r. spectrum, with resonances of the 'benzoyl' groups spread over the range δ 7.16–8.26. Despite the apparent similarity of environment of these two phenyl rings, the chemical shifts of the two pairs of *ortho* protons were widely different at δ 7.40 and 8.26. A two-dimensional technique (COSY 45) grouped the former with the

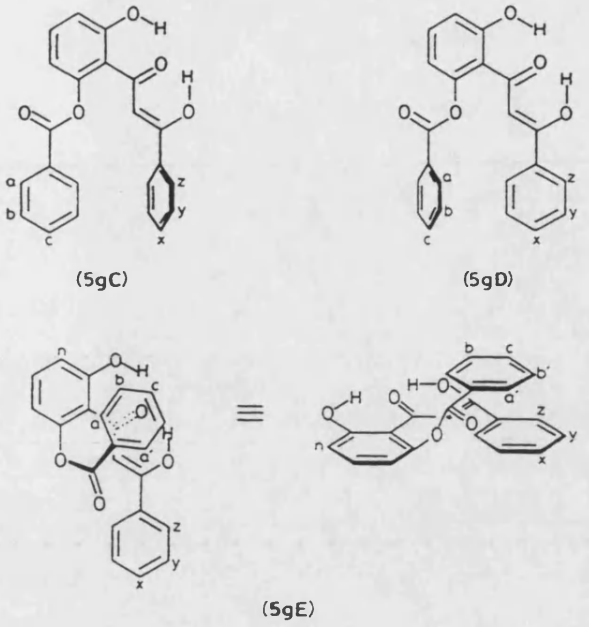


Figure 3. Possible conformations of (5g).

meta proton resonances at δ 7.16 and the *para* proton resonance at δ 7.37. Similarly, the signals at δ 7.52 and 7.68 were shown to arise from the same phenyl ring as downfield *ortho* proton resonances. The chemical shift of the 'downfield' set of signals is unremarkable but that of the 'upfield' set is unusual. The orientation of the phenyl rings in the molecule should explain the shielding of one of the rings seen in the spectrum. There are a number of potential orientations of the two phenyl rings and these are shown in Figure 3.

The first conformation (5gC) shows the *x,y,y′,z,z′* ring to be at a steep angle to the enol system and so the *z,z′* protons may be shielded by loss of conjugation. The second orientation (5gD) shows the *a,a′,b,b′,c* phenyl ring at a steep angle to the carbonyl group, shielding the *a,a′* protons. The third possible orientation (5gE) shows the benzoate ester to be positioned so that the phenyl ring lies over the enol system in a 'coiled' molecular conformation. In this last orientation, the *a,a′* protons would be shielded by interaction with the π-orbitals of the enol system.

In order to verify which, if any, of these possibilities is correct, a number of n.m.r. experiments were performed. If the *z,z′* phenyl ring is in plane with the enol system [as in (5gD)], then the *z,z′* protons would be expected to experience a nuclear Overhauser effect (n.O.e.) from the vinylic proton. Irradiation of the proton at δ 6.87 did indeed produce an n.O.e., however both sets of protons *ortho* to the carbonyl groups showed an enhancement, although there was a fivefold enhancement of the resonances at δ 7.4 compared with that at δ 8.3. This suggests that the protons generating the upfield signal are closer in space to the vinyl protons than the protons generating the downfield signal, although these protons are also close enough to the vinyl proton to experience some small n.O.e. This experiment rules out one of the potential orientations of the phenyl rings (5gD). If the *a,a′* phenyl ring were normal to the carboxylate system then no n.O.e. would be experienced by the *a,a′* protons when the vinyl proton was irradiated.

If the *z,z′* phenyl ring were at right angles to the carbonyl system [as in (5gC)], then no n.O.e. would be expected. If, however, this phenyl ring were co-planar with the enol system then a large n.O.e. should be seen but this does not explain why

these protons would be shielded. Consideration of the crystal structure of 1-(2-hydroxy-4-methoxyphenyl)-3-phenylpropane-1,3-dione shows that the phenyl ring attached to the 3-carbon in (**5a**) is out of plane by 14°, so this may explain why an n.O.e. is seen at both the *z,z'* and the *a,a'* protons.

To determine if the *a,b,c* phenyl ring is orientated in such a way that it lies over the top of the enol system [as in (**5gE**)], another n.O.e. difference experiment was carried out. This time the enolic OH, at δ 15.5, was irradiated. This, too, produced an ambiguous result, in that saturation transfer to the phenolic OH caused enhancement of the resonance due to the *ortho* proton. However, by far the largest n.O.e. enhancement observed was again at the *ortho* doublet of the upfield set of protons, although the downfield *ortho* protons also appeared to be stimulated but to a much smaller extent. Although none of these spectroscopic data are completely conclusive, the balance of evidence would tend to support the 'coiled' conformation (**5gE**) as representing the structure of the enol form of (**5g**).

The results of the spectroscopic experiments described above show that extensive enolisation of the majority of the substituted 1-(2-hydroxyphenyl)-3-phenylpropane-1,3-diones (**5**) is facile in solution in deuteriochloroform. Crystallographic evidence on (**5a, b**) illustrates that, depending on substitution patterns, these compounds can crystallise either in the keto or enol form.

Experimental

I.r. spectra were recorded on a Perkin-Elmer 1310 spectrometer. ^{1}H N.m.r. spectra were recorded at 60 MHz using a Varian EM360A instrument, at 300 MHz using a Bruker AC300 instrument, and at 400 MHz with a Bruker WH400 spectrometer; samples were dissolved in $CDCl_3$ and tetramethylsilane was used as an internal standard. Mass spectra were obtained using a VG Micromass 12B single focussing mass spectrometer in the electron-impact mode. Light petroleum refers to the fraction b.p. 60–80 °C.

1-Acetyl-4,6-dimethoxy-2-hydroxybenzene (**2b**).—3,5-Dimethoxyphenol (**1b**) (7.7 g, 50 mmol) in dichloromethane (50 cm^3) was added to boron trichloride in dichloromethane (1 mol dm^{-3}; 50 cm^3) under nitrogen at −10 °C. The mixture was stirred for 5 min and acetyl chloride (3.9 g, 50 mmol) was added. The reaction mixture was then heated to reflux for 3 h before being carefully quenched by addition of an excess of hydrochloric acid (1 mol dm^{-3}). After 1 h at ambient temperature, the organic phase was separated and the solvent was evaporated under reduced pressure. The residue, in dichloromethane (50 cm^3), was filtered through a silica plug. Removal of the solvent from the filtrate under reduced pressure afforded 1-acetyl-2-hydroxy-4,6-dimethoxybenzene (7.7 g, 78%) as a white solid, m.p. 82 °C (lit.,[11] 82–83 °C); δ_H(60 MHz) 2.56 (3 H, s, COMe), 3.78 (3 H, s, OMe), 3.80 (3 H, s, OMe), 5.85 (1 H, d, *J* 2 Hz, ArH), 6.00 (1 H, d, *J* 2 Hz, ArH), and 13.9 (1 H, s, OH).

1-Acetyl-2-hydroxy-4-methoxybenzene (**2a**).—3-Methoxyphenol (**1a**) was treated with boron trichloride and acetyl chloride, as for the preparation of (**2b**) above, to afford 1-acetyl-2-hydroxy-4-methoxybenzene (78%) as white crystals, m.p. 50 °C (lit.,[12] 48 °C); δ_H(60 MHz) 2.50 (3 H, s, COMe), 3.75 (3 H, s, OMe), 6.30 (1 H, d, *J* 2 Hz, 6-H), 6.37 (1 H, dd, *J* 9, 2 Hz, 4-H), 7.54 (1 H, d, *J* 9 Hz, 3-H), and 12.72 (1 H, s, OH).

2-Acetyl-3,5-dimethoxyphenyl Benzoate (**4b**).—2-Acetyl-3,5-dimethoxyphenol (**2b**) (2.94 g, 15 mmol) was treated with benzoyl chloride (**3a**) (2.8 g, 20 mmol), and 4-(dimethylamino)pyridine (366 mg, 3 mmol) in pyridine (15 cm^3) at ambient temperature for 10 min. The mixture was poured onto an excess of hydrochloric acid (1 mol dm^{-3}) and crushed ice. The solid was collected by filtration and recrystallised from chloroform–light petroleum to furnish *2-acetyl-3,5-dimethoxyphenyl benzoate* (3.16 g, 70%) as white crystals, m.p. 92–93 °C (Found: C, 67.7; H, 5.4. $C_{17}H_{16}O_5$ requires C, 68.0; H, 5.35%); ν_{max}(Nujol) 1 735 and 1 680 cm^{-1}; δ_H(60 MHz) 2.41 (3 H, s, COMe), 3.73 (3 H, s, OMe), 3.75 (3 H, s, OMe), 6.32 (2 H, s, 4,6-H), 7.3–7.7 (3 H, m, 3',4',5'-H), and 8.05 (2 H, dd, *J* 7, 2 Hz, 2',6'-H); *m/z* 300 (*M*$^+$), 285, 105 (100%), and 77.

2-Acetylphenyl Benzoate (**4c**).—2-Acetylphenol (**2c**) was treated with benzoyl chloride (**3a**) as for the preparation of (**4b**) above, except that the dimethylaminopyridine was omitted and the solvent for recrystallisation was methanol, to afford 2-acetylphenyl benzoate (78%) as a white solid, m.p. 89–90 °C (lit.,[6] 87–88 °C); δ_H(60 MHz) 2.4 (3 H, s, COMe), 7.0–7.6 (6 H, m, 3',4,4',5,5',6-H), 7.70 (1 H, dd, *J* 8, 2 Hz, 3-H), and 8.15 (2 H, dd, *J* 6, 2 Hz, 2',6'-H).

2-Acetyl-5-methoxyphenyl Benzoate (**4a**).—1-Acetyl-2-hydroxy-4-methoxybenzene (**2a**) was treated with benzoyl chloride (**3a**) as for the preparation of (**4b**) above, with the exception of recrystallisation, to give *2-acetyl-5-methoxyphenyl benzoate* (84%) as a pale yellow oil; ν_{max} (liquid film) 1 735 and 1 680 cm^{-1}; δ_H(60 MHz) 2.45 (3 H, s, COMe), 3.78 (3 H, s, Me), 6.70 (1 H, d, *J* 2 Hz, 6-H), 6.75 (1 H, dd, *J* 8, 2 Hz, 4-H), 7.53 (3 H, m, 3',4',5'-H), 7.81 (1 H, d *J* 8 Hz, 3-H), and 8.15 (2 H, dd, *J* 9, 2 Hz, 2',6'-H); *m/z* 270.0891 (*M*$^+$) ($C_{16}H_{14}O_4$ requires 270.0892), 105 (100%), and 77.

2-Acetyl-3,5-dimethoxyphenyl 4-Nitrobenzoate (**4d**).—1-Acetyl-4,6-dimethoxy-2-hydroxybenzene (**2b**) was treated with 4-nitrobenzoyl chloride (**3b**) as for the preparation of (**4b**) above, except that ethyl acetate–light petroleum was used as the solvent for recrystallisation, to give *2-acetyl-3,5-dimethoxyphenyl 4-nitrobenzoate* (67%) as pale yellow crystals, m.p. 159–160 °C (Found: C, 59.15; H, 4.35; N, 4.05. $C_{17}H_{15}NO_7$ requires C, 59.15; H, 4.35; N, 4.05%); δ_H(60 MHz) 2.45 (3 H, s, COMe), 3.80 (3 H, s, OMe), 3.86 (3 H, s, OMe), 6.31 (1 H, d, *J* 2 Hz, 6-H), 6.38 (1 H, d, *J* 2 Hz, 4-H), and 8.26 (4 H, s, 2',3',5',6'-H); *m/z* 345 (*M*$^+$), 330 (100%), 314, 286, 256, 150, 137, 104, and 76.

2-Acetyl-5-methoxyphenyl 4-Nitrobenzoate (**4e**).—2-Acetyl-1-hydroxy-5-methoxybenzene (**2a**) was treated with 4-nitrobenzoyl chloride (**3b**) as for the preparation of (**4b**) above to afford 2-acetyl-5-methoxyphenyl 4-nitrobenzoate (68%) as a pale yellow solid, m.p. 141 °C (lit.,[13] 135–137 °C); δ_H(60 MHz) 2.45 (3 H, s, COMe), 3.83 (3 H, s, OMe), 6.66 (1 H, d, *J* 2 Hz, 6-H), 6.76 (1 H, dd, *J* 9, 2 Hz, 4-H), 7.80 (1 H, d, *J* 9 Hz, 3-H), and 8.28 (4 H, s, 2',3',5',6'-H).

1-Acetyl-2,4-bis(benzoyloxy)benzene (**4f**).—1-Acetylbenzene-2,4-diol (**2d**) (7.6 g, 50 mmol) was treated with benzoyl chloride (**3a**) (16.8 g, 120 mmol) in pyridine (20 cm^3) for 1 h and the mixture was poured onto an excess of hydrochloric acid (1 mol dm^{-3}) and ice. The solid was collected by filtration and recrystallised from methanol to furnish *1-acetyl-2,4-bis(benzoyloxy)benzene* (14.94 g, 83%) as a white solid, m.p. 85 °C (Found: C, 73.25; H, 4.45. $C_{22}H_{16}O_5$ requires C, 73.3; H, 4.45%); δ_H(60 MHz) 2.53 (3 H, s, COMe), 7.18 (1 H, d, *J* 2 Hz, 6-H), 7.37 (1 H, dd, *J* 8, 2 Hz, 4-H), 7.40–7.63 (6 H, m, 3',4',5',3'',4'',5''-H), 7.90 (1 H, d, *J* 8 Hz, 3-H), and 8.06–8.26 (4 H, m, 2',6',2'',6''-H); *m/z* 360 (*M*$^+$), 105 (100%), 77, 52, and 43.

2-Acetyl-1,3-bis(benzoyloxy)benzene (**4g**).—2-Acetylbenzene-1,3-diol (**2e**) was treated with benzoyl chloride (**3a**) as for the preparation of (**4e**) above to afford *2-acetyl-1,3-bis(benzoyloxy)*-

benzene (63%) as a white solid, m.p. 108 °C (Found: C, 73.3; H, 4.6. $C_{22}H_{16}O_5$ requires C, 73.3; H, 4.45%); δ_H(60 MHz) 2.45 (3 H, s, COMe), 7.1–7.7 (9 H, m, 4,5,6,3′,4′,5′,3″,4″,5″-H), and 8.14 (4 H, m, 2′,2″, 6′,6″-H); *m/z* 360 (M^+), 105 (100%), 77, 52, and 43.

2-*Acetylphenyl* 3,4-*Dichlorobenzoate* (**4h**).—2-Acetylphenol (**2c**) was treated with 3,4-dichlorobenzoyl chloride (**3c**) as for the preparation of (**4e**) above to afford 2-*acetylphenyl* 3,4-*dichlorobenzoate* (78%) as a white solid, m.p. 84 °C (Found: C, 58.1; H, 3.25. $C_{15}H_{10}Cl_2O_3$ requires C, 58.25; H, 3.25%); δ_H(60 MHz) 2.52 (3 H, s, COMe), 7.15 (1 H, dd, *J* 8, 2 Hz, 6′-H), 7.30–7.50 (2 H, m, 4,5-H), 7.56 (1 H, d, *J* 8 Hz, 5′-H), 7.83 (1 H, dd, *J* 8, 2 Hz, 3-H), 7.92 (1 H, dd, *J* 8, 2 Hz, 6′-H), and 8.26 (1 H, d, *J* 2 Hz, 2′-H); *m/z* 308/310/312 (M^+), 173/175/177 (100%), 145/145/149, and 75.

2-*Acetylphenyl* 3,4-*Dimethoxybenzoate* (**4i**).—2-Acetylphenol (**2c**) was treated with 3,4-dimethoxybenzoyl chloride (**3d**) as for the preparation of (**4e**) above to afford 2-acetylphenyl 3,4-dimethoxybenzoate (84%) as a white solid, m.p. 126–127 °C (lit.,[14] 129 °C) (Found: C, 68.0; H, 5.4. $C_{17}H_{16}O_5$ requires C, 68.0; H, 5.35%); δ_H(60 MHz) 2.5 (3 H, s, COMe), 3.92 (6 H, s, 2 × OMe), and 6.8–7.9 (7 H, m, ArH).

2-*Acetylphenyl* 4-*Chlorobenzoate* (**4j**).—2-Acetylphenol (**2c**) was treated with 4-chlorobenzoyl chloride (**3e**) as for the preparation of (**4e**) above to yield 2-acetylphenyl 4-chlorobenzoate (75%) as a white solid, m.p. 94 °C (lit.,[15] 92–93 °C) (Found: C, 65.65; H, 4.2. $C_{15}H_{11}ClO_3$ requires C, 65.6; H, 4.05%); δ_H(60 MHz) 2.50 (1 H, s, COMe), 7.1–7.4 (3 H, m, 4,5,6-H), 7.42 (2 H, d, *J* 8 Hz, 3′,5′-H), 7.80 (1 H, dd, *J* 7 and 1.5 Hz, 3-H), and 8.08 (2 H, d, *J* 8 Hz, 2′,6′-H).

2-*Acetylphenyl* 4-*Nitrobenzoate* (**4k**).—2-Acetylphenol (**2c**) was treated with 4-nitrobenzoyl chloride (**4b**) as for the preparation of (**4e**) above to yield 2-acetylphenyl 4-nitrobenzoate (65%) as a pale yellow solid, m.p. 90 °C (lit.,[16] 99–100 °C); δ_H(60 MHz) 2.50 (3 H, s, COMe), 7.10–7.67 (3 H, m, 4,5,6-H), 7.88 (1 H, dd, *J* 6, 2 Hz, 3-H), and 8.36 (4 H, s, 2′,3′,5′,6′-H).

1-(2-*Hydroxy*-4,6-*dimethoxyphenyl*)-3-*phenylpropane*-1,3-*dione* (**5b**).—Butyl-lithium (1.44 mol dm^{-3} in hexane; 14 cm^3, 20 mmol) was added to hexamethyldisilazane (2.82 g, 20 mmol) in anhydrous tetrahydrofuran (30 cm^3) at −78 °C under nitrogen. 2-Acetyl-3,5-dimethoxyphenyl benzoate (**4b**) (2.25 g, 10 mmol) in anhydrous tetrahydrofuran (20 cm^3) was then added during 15 min. The mixture was allowed to warm to 20 °C and was quenched with an excess of hydrochloric acid (1 mol dm^{-3}). The solid was collected by filtration and washed with water before being recrystallised from methanol to yield 1-(2-*hydroxy*-4,6-*dimethoxyphenyl*)-3-*phenylpropane*-1,3-*dione* (1.42 g, 63%) as a yellow solid, m.p. 151 °C (Found: C, 67.95; H, 5.30. $C_{17}H_{16}O_5$ requires C, 68.0; H, 5.35%); ν_{max}(Nujol) 2 700 br and 1 600 cm^{-1}; δ_H(300 MHz) 3.43 (3 H, s, OMe), 3.78 (3 H, s, OMe), 4.51 (2 H, s, $COCH_2CO$), 5.78 (1 H, d, *J* 2 Hz, 3′-H), 6.02 (1 H, d, *J* 2 Hz, 5′-H), 7.4–7.6 (3 H, m, 3″,4″,5″-H), 7.8–8.0 (2 H, m, 2″,6″-H), and 13.67 (1 H, s, OH); *m/z* 300 (M^+), 283, 269, 181, 105 (100%), and 77.

1-(2-*Hydroxy*-4-*methoxyphenyl*)-3-(4-*nitrophenyl*)*propane*-1,3-*dione* (**5e**).—2-Acetyl-5-methoxyphenyl 4-nitrobenzoate (**4e**) was treated with lithium hexamethyldisilazide as for the synthesis of (**5b**) above to give 1-(2-*hydroxy*-4-*methoxyphenyl*)-3-(4-*nitrophenyl*)*propane*-1,3-*dione* (48%) as yellow hexagonal crystals, m.p. 200 °C (Found: C, 60.7; H, 4.15; N, 4.45. $C_{16}H_{13}NO_6$ requires C, 60.95; H, 4.15; N, 4.45%); δ_H(300 MHz) 3.81 (3 H, s, OCH_3), 4.61 (0.16 H, s, $COCH_2CO$), 6.48 (1 H, d, *J* 2.4 Hz, 3′-H), 6.51 (1 H, dd, *J* 8.9, 2.4 Hz, 5′-H), 6.78 (0.92 H, s, vinyl-H), 7.70 (1 H, d, *J* 8.9 Hz, 6′-H), 8.08 (2 H, d, *J* 8.8 Hz, 2″,6″-H), 8.33 (2 H, d, *J* 8.8 Hz, 3″,5″-H), 12.20 [0.08 H, s, phenolic OH (keto form)], 12.44 [0.92 H, s, phenolic OH (enol form)], and 15.20 (0.92 H, s, enol OH); *m/z* 315 (M^+), 298, 285, 193, 150 (100%), 104, and 76.

1-(2-*Hydroxy*-4-*methoxyphenyl*)-3-*phenylpropane*-1,3-*dione* (**5a**).—2-Acetyl-5-methoxyphenyl benzoate (**4a**) was treated with lithium hexamethyldisilazide as for the synthesis of (**5b**) above to give 1-(2-*hydroxy*-4-*methoxyphenyl*)-3-*phenylpropane*-1,3-*dione* (37%) as brown transparent crystals, m.p. 138 °C (Found: C, 71.1; H, 4.95. $C_{16}H_{14}O_4$ requires C, 71.1; H, 5.2%); ν_{max}(Nujol) 2 700 br, 1 680, and 1 580 cm^{-1}; δ_H(300 MHz) 3.76 (3 H, s, OMe), 4.5 (0.16 H, brs, $COCH_2CO$), 6.42 (2 H, dd, *J* 9, 2 Hz, 5′-H), 6.53 (1 H, d, *J* 2 Hz, 3′-H), 6.62 (0.88 H, s, vinyl-H), 7.4–7.6 (3 H, m, 3″,4″,5″-H), 7.60 (1 H, d, *J* 9 Hz, 6-H), 7.7–7.9 (2 H, m, 2″,6″-H), 12.50 (1 H, s, phenolic OH), and 15.40 (0.88 H, s, enol OH); *m/z* 300 (M^+), 283, 269, 181, 105 (100%), and 77.

1-(2-*Hydroxy*-4,6-*dimethoxyphenyl*)-3-(4-*nitrophenyl*)-*propane*-1,3-*dione* (**5d**).—2-Acetyl-3,5-dimethoxyphenyl 4-nitrobenzoate (**4d**) was treated with lithium hexamethyldisilazide as for the synthesis of (**5b**) above to give 1-(2-*hydroxy*-4,6-*dimethoxyphenyl*)-3-(4-*nitrophenyl*)*propane*-1,3-*dione* (53%) as a bright yellow solid, m.p. 192 °C (Found: C, 59.05; H, 4.4; N, 3.85. $C_{17}H_{15}NO_7$ requires C, 59.15; H, 4.35; N, 4.05%); ν_{max}(Nujol) 2 700–3 100 br, 1 575, 1 515, and 1 340 cm^{-1}; δ_H(300 MHz) 3.53 [0.6 H, s, OMe (keto)], 3.82 [0.6 H, s, OMe (keto)], 3.85 [2.4 H, s, OMe (enol)], 3.94 [2.4 H, s, OMe (enol)], 4.59 (0.4 H, s, $COCH_2CO$), 5.87 [0.2 H, d, *J* 2.4 Hz, 5′-H (keto)], 6.06 [0.8 H, d, *J*, 2.4 Hz, 5′-H (enol)], 6.11 [0.2 H, d, *J* 2.4 Hz, 3′-H (keto)], 6.13 [0.8 H, d, *J* 2.4 Hz, 3′-H (enol)], 7.41 (0.8 H, s, vinyl-H), 8.03 [1.6 H, d, *J* 8.9 Hz, 2″,6″-H (enol)], 8.13 [0.4 H, d, *J* 8.9 Hz, 2″,6″-H (keto)], 8.32 [1.6 H, d, *J* 8.9 Hz, 3″,5″-H (enol)], 8.36 [0.4 H, H, d, *J* 8.9 Hz, 3″,5″-H (keto)], 13.33 [0.8 H, s, phenolic OH (enol)], 13.55 [0.2 H, s, phenolic OH (keto)], and 15.26 (0.8 H, s, enol OH).

1-(2-*Hydroxyphenyl*)-3-*phenylpropane*-1,3-*dione* (**5c**).—Finely powdered potassium hydroxide (1.7 g, 30 mmol) was added to 2-acetylphenyl benzoate (**4c**) (4.8 g, 20 mmol) in pyridine (50 cm^3) at 50 °C. The mixture was stirred for 20 min before being poured into a slight excess of hydrochloric acid (1 mol dm^{-3}). The solid was collected by filtration and was recrystallised from methanol to give 1-(2-hydroxyphenyl)-3-phenylpropane-1,3-dione (1.07 g, 63%) as a yellow solid, m.p. 124 °C (lit.,[17] 117–120 °C); δ_H(400 MHz) 4.63 (0.13 H, s, $COCH_2CO$), 6.84 (0.94 H, s, vinyl-H), 6.92 (1 H, ddd, *J* 8.0, 7.0, 1.1 Hz, 5′-H), 7.00 (1 H, dd, *J* 8.3, 1.1 Hz, 3′-H), 7.46 (1 H, ddd, *J* 8.3, 7.0, 1.1 Hz, 4′-H), 7.48 (2 H, dd, *J* 8.0, 7.3 Hz, 3″,5″-H), 7.56 (1 H, tt, *J* 7.3, 1.6 Hz, 4″-H), 7.79 (1 H, dd, *J* 8.0, 1.6 Hz, 6′-H), 7.94 (2 H, dd, *J* 8.0, 1.6 Hz, 2″,6″-H), 11.93 [0.06 H, s, phenolic OH (keto)], 12.10 [0.94 H, s, phenolic OH (enol)], and 15.54 (0.94 H, s, enol OH).

1-(4-*Benzoyloxy*-2-*hydroxyphenyl*)-3-*phenylpropane*-1,3-*dione* (**5f**).—1-Acetyl-2,4-bis(benzoyloxy)benzene (**4f**) was treated with potassium hydroxide in pyridine as for the preparation of (**5c**) above to give 1-(4-benzoyloxy-2-hydroxyphenyl)-3-phenylpropane-1,3-dione (52%) as yellow crystals, m.p. 171 °C (lit.,[18] 167 °C); δ_H(400 MHz) 4.63 (0.2 H, s, $COCH_2CO$), 6.80 (0.9 H, s, vinyl-H), 6.84 (1 H, dd, *J* 8.9, 2.3 Hz, 5′-H), 6.89 (1 H, d, *J* 2.3 Hz, 3′-H), 7.5 [5 H, m, 3‴,3‴, (4‴-H or 4‴′-H), 5″,5‴′-H], 7.66 (1 H, tt, *J* 7.4, 1.3 Hz, 4″-H or 4‴′-H), 7.85 (1 H, d, *J* 8.9 Hz, 6′-H), 7.94 [2 H, dd, *J* 8.4, 1.3 Hz, (2″,6″-H) or (2‴,6‴′-H)], 8.19 [2 H, dd, *J* 8.4, 1.2 Hz, (2″,6″-

H) or (2‴,6‴-H)], 12.16 [0.1 H, s, phenolic OH (keto)], 12.36 [0.9 H, s, phenolic OH (enol)] and 15.45 (0.9 H, s, enol OH).

1-(6-*Benzoyloxy*-2-*hydroxyphenyl*)-3-*phenylpropane*-1,3-*dione* (**5g**).—2-Acetyl-1,3-bis(benzoyloxy)benzene (**4g**) was treated with potassium hydroxide in pyridine as for the preparation of (**5c**) above to give 1-(6-*benzoyloxy*-2-*hydroxyphenyl*)-3-*phenylpropane*-1,3-*dione* (64%) as yellow crystals, m.p. 184 °C (Found: C, 73.4; H, 4.5. $C_{22}H_{16}O_5$ requires C, 73.3; H, 4.45%); δ_H(400 MHz) 6.75 (1 H, dd, *J* 7.8, 0.9 Hz, 3′-H or 5′-H), 6.87 (1 H, s, vinyl-H), 6.95 (1 H, dd, *J* 8.5, 0.9 Hz, 3′-H or 5′-H), 7.16 (2 H, dd, *J* 8.1, 7.6 Hz, 3‴,5‴-H), 7.37 (1 H, tt, *J* 7.3, 1.2 Hz, 4‴-H), 7.40 (2 H, dd, *J* 7.3, 1.2 Hz, 2‴,6‴-H), 7.45 (1 H, t, *J* 8.2 Hz, 4′-H), 7.52 (2 H, dd, *J* 7.9, 7.6 Hz, 3″,5″-H), 7.68 (1 H, tt, *J* 7.4, 1.2 Hz, 4″-H), 8.26 (2 H, dd, *J* 8.3, 1.2 Hz, 2″,6″-H), 11.91 (1 H, s, phenolic OH), and 15.73 (1 H, s, enol OH); *m/z* 260 (M^+), 238, 105 (100%), and 77.

1-(3,4-*Dichlorophenyl*)-3-(2-*hydroxyphenyl*)*propane*-1,3-*dione* (**5h**).—2-Acetylphenyl 3,4-dichlorobenzoate (**4h**) was treated with potassium hydroxide in pyridine as for the preparation of (**5c**) above to give 1-(3,4-*dichlorophenyl*)-3-(2-*hydroxyphenyl*)*propane*-1,3-*dione* (47%) as a yellow solid, m.p. 155–157 °C (Found: C, 58.1; H, 3.2. $C_{15}H_{10}Cl_2O_3$ requires C, 58.25; H, 3.25%); δ_H(400 MHz) 4.59 (0.14 H, s, $COCH_2CO$), 6.78 (0.93 H, s, vinyl-H), 6.93 (1 H, ddd, *J* 8.16, 7.1, 1.0 Hz, 5′-H), 7.00 (1 H, dd, *J* 8.4, 1.0 Hz, 3′-H), 7.48 (1 H, ddd, *J* 8.4, 7.0, 1.5 Hz, 4′-H), 7.56 (1 H, d, *J* 8.5 Hz, 5″-H), 7.75 (1 H, dd, *J* 8.5, 2.1 Hz, 6″-H), 7.77 (1 H, dd, *J* 8.2, 1.5 Hz, 6′-H), 8.00 (1 H, d, *J* 2.1 Hz, 2″-H), 11.80 [0.07 H, s, phenolic OH (keto)], 11.95 [0.93 H, s, phenolic OH (enol)], and 15.43 (0.93 H, s, enol OH); *m/z* 308/310/312 (M^+), 173/175/177 (100%), and 121.

1-(3,4-*Dimethoxyphenyl*)-3-(2-*hydroxyphenyl*)*propane*-1,3-*dione* (**5i**).—2-Acetylphenyl 3,4-dimethoxybenzoate (**4i**) was treated with potassium hydroxide in pyridine as for the preparation of (**5c**) above to give 1-(3,4-dimethoxyphenyl)-3-(2-hydroxyphenyl)propane-1,3-dione (62%) as a yellow solid, m.p. 115 °C (lit.,[19] 115 °C); δ_H(400 MHz) 3.90 [0.63 H, s, OMe (keto)], 3.94 [0.63 H, s, OMe (keto)], 3.95 [2.37 H, s, OMe (enol)], 3.96 [2.37 H, s, OMe (enol)], 4.58 (0.42 H, s, $COCH_2CO$), 6.75 (0.79 H, s, vinyl-H), 6.90 [0.79 H, ddd, *J* 8.1, 7.0, 1.0 Hz, 5′-H (enol)], 6.91 [0.21 H, ddd, *J* 8.0, 7.0, 1.0 Hz, 5′-H (keto)], 6.91 [0.21 H, d, *J* 8.5 Hz, 5″-H (keto)], 6.92 [0.79 H, d, *J* 8.5 Hz, 5-H (enol)], 6.97 (0.21 H, dd, *J* 8.6, 1.0 Hz, 3′-H (keto)], 6.98 (0.79 H, dd, *J* 8.4, 1.0 Hz, 3′-H (enol)], 7.43 (0.79 H, ddd, *J* 8.4, 7.0, 1.5 Hz, 4′-H (enol)], 7.44 (1 H, d, *J* 2.1 Hz, 2″-H), 7.47 (0.21 H, ddd, *J* 8.6, 7.0, 1.6 Hz, 4′-H (keto)], 7.56 [0.79 H, dd, *J* 8.5, 2.1 Hz, 6″-H (enol)], 7.63 [0.21 H, dd, *J* 8.3, 2.0 Hz, 6″-H (keto)], 7.75 [0.79 H, dd, *J* 8.1, 1.5 Hz, 6′-H (enol)], 7.80 [0.21 H, dd, *J* 8.0, 1.6 Hz, 6′-H (keto)], 11.96 [0.21 H, s, phenolic OH (keto)], 12.09 [0.79 H, s, phenolic OH (enol)], and 15.85 (0.79 H, s, enol OH).

1-(4-*Chlorophenyl*)-3-(2-*hydroxyphenyl*)*propane*-1,3-*dione* (**5j**).—2-Acetylphenyl 4-chlorobenzoate (**4j**) was treated with potassium hydroxide in pyridine as for the preparation of (**5c**) above to give 1-(2-hydroxyphenyl)-3-(4-chlorophenyl)propane-1,3-dione (43%) as yellow crystals, m.p. 120 °C (lit.,[15] 122.4 °C); δ_H(60 MHz) 6.70 (1 H, s, vinyl-H), 6.80–7.40 (3 H, m, 3′,4′,5′-H), 7.37 (2 H, d, *J* 8 Hz, 3″-H), 7.66 (1 H, dd, *J* 7, 1 Hz, 6′-H), 7.79 (2 H, d, *J* 8 Hz, 2″-H), 11.95 (1 H, s, phenolic OH), and 15.5 (1 H, brs, enol OH).

1-(2-*Hydroxyphenyl*)-3-(4-*nitrophenyl*)*propane*-1,3-*dione* (**5k**).—2-Acetylphenyl 4-nitrobenzoate (**4k**) was treated with potassium hydroxide in pyridine as for the preparation of (**5c**) above to give 1-(2-hydroxyphenyl)-3-(4-nitrophenyl)propane-1,3-dione (58%) as a yellow solid, m.p. 195 °C (lit.,[16] 198–201 °C); δ_H(400 MHz) 6.90 (1 H, s, vinyl-H), 6.95 (1 H, ddd, *J* 8.1, 7.1, 1.0 Hz, 5′-H), 7.03 (1 H, dd, *J* 8.5, 1.0 Hz, 3′-H), 7.51 (1 H, ddd, *J* 8.5, 7.1, 1.6 Hz, 4′-H), 7.79 (1 H, dd, *J* 8.1, 1.6 Hz, 6′-H), 8.09 (2 H, d, *J* 8.9 Hz, 2″,6″-H), 8.33 (2 H, d, *J* 8.9 Hz, 3″,5″-H), 11.92 (1 H, s, phenolic OH), and 15.30 (1 H, s, enol OH).

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Labelled Compounds of Interest as Antitumour Agents. Part II. Synthesis of ^{2}H and ^{3}H Isotopomers of RSU 1069 and Ro 03-8799 (Pimonidazole)

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LABELLED COMPOUNDS OF INTEREST AS ANTITUMOUR AGENTS. PART II (1). SYNTHESIS OF ^{2}H AND ^{3}H ISOTOPOMERS OF RSU 1069 AND Ro 03-8799 (PIMONIDAZOLE).

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SUMMARY

^{2}H- and ^{3}H-Labelled RSU 1069 and Ro 03-8799 (pimonidazole) have been synthesised by reduction of 1-(3-chloro-2-oxopropyl)-2-nitroimidazole with the appropriately labelled sodium borohydride, followed by ring-closure of the chlorohydrins and treatment of the resulting epoxides with aziridine or piperidine. In both cases, the specific activities were 200 mCi $mmol^{-1}$ and the radiochemical yields were 86 %. The parent compounds are radiosensitisers of hypoxic tumour cells.

Key words: Radiosensitiser, Pimonidazole. [^{2}H]-Ro 03-8799, [^{3}H]-Ro 03-8799, [^{2}H]-RSU 1069, [^{3}H]-RSU 1069.

INTRODUCTION

RSU 1069 (1) (2-4) and pimonidazole (Ro 03-8799, 2) (5,6) are members of the second generation of electron-affinic radiosensitisers based on 2-nitroimidazole and have shown some useful additional activity and/or lower toxicity when compared with the archetype misonidazole (3) (Figure 1). The aziridine 1 also exhibits much greater selective toxicity towards hypoxic cells than does misonidazole (7-9), this selectivity arising from the ability of the compound to act as a bifunctional electrophile under hypoxia (10-12). [^{14}C]-RSU 1069, prepared from 2-[^{14}C]-2-nitroimidazole, has been shown to react with DNA in

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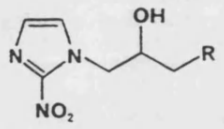
vitro (11). A study (13) of the pharmacokinetics and metabolism of 1, using unlabelled material, has revealed several metabolites of low molecular weight, although this technique does not give information on the nature and location of chemical and metabolic processes involving persistent covalent binding to biological macromolecules. It was therefore of interest to prepare a tritium labelled isotopomer of 1 at moderate specific activity to enable the extension of these studies in vitro and in vivo. The synthetic route employed also facilitated the preparation of a radiolabelled isotopomer of 2 for analogous studies, although electrophilic reaction of this agent with macromolecules would not be expected in the absence of bioreduction.



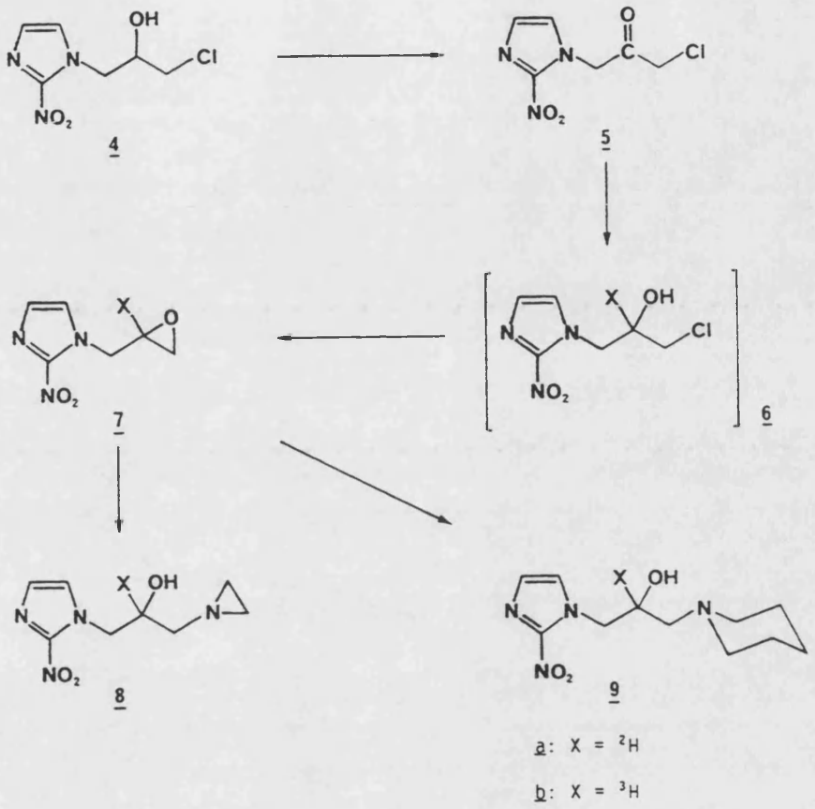
1: R = aziridin-1-yl

2: R = piperidin-1-yl

3: R = OMe

FIGURE 1.

[^{3}H]-Misonidazole has been reported (14,15) to be formed by the reduction of the corresponding ketone (1-(3-methoxy-2-oxopropyl)-2-nitroimidazole). This most direct incorporation of tritium into RSU 1069 by reduction of the corresponding aziridinylmethylketone could not, however, be achieved since attempts to oxidise the secondary alcohol function of 1 resulted in concomitant destruction of the highly labile aziridine moiety. However, oxidation of the chlorohydrin 4 (16) with chromium trioxide in acidic acetone furnished the ketone 5 in modest yield (Scheme 1) in a reaction similar to that reported (16) for the oxidation of 3. Treatment of 5 with 0.29 molar equivalents of sodium boro[^{2}H]hydride gave complete reduction as shown by thin layer chromatography (TLC), indicating that all four hydrogens of the borohydride are available for reaction with this highly electrophilic carbonyl group. The intermediate [^{2}H]-chlorohydrin 6a was not isolated owing to its poor solubility in organic solvents but was cyclised in the presence of aqueous base to give the readily isolable [^{2}H]-epoxide 7a.



SCHEME 1.

In order to investigate whether primary kinetic isotope effects may cause significant preferential incorporation of ^{1}H rather than ^{3}H from sodium boro[^{3}H]hydride, 5 was treated with an excess of a mixture of $NaB^{1}H_4$ and $NaB^{2}H_4$. Subsequent cyclisation gave a mixture of the [^{1}H]- and [^{2}H]-epoxides. From the integral of the multiplet centred at δ 3.44 in the ^{1}H NMR spectrum (from the 2-H of the side-chain), it was estimated that $k[^{1}H]/k[^{2}H] = 1.5$ for the reduction step. Therefore, to avoid poor incorporation due to a significant value of $k[^{1}H]/k[^{3}H]$, an excess of 5 was treated with sodium boro[^{3}H]hydride of high specific activity, followed after a short period by an excess of $NaB^{1}H_4$ to complete the reduction. Again, the chlorohydrin 6b was not isolated but converted to the [^{3}H]-epoxide 7b in near quantitative chemical and radiochemical yield. Thus this 'cold chaser' procedure offers an advantage in incorporation of radiolabel when compared with the lower labelling reported by

Setiabaudi et al (17) for the analogous stoichiometric reduction/cyclisation of phenacyl bromides to phenylepoxides.

The epoxides 7a,b were converted in essentially quantitative chemical and radiochemical yields to the required aziridines 8a,b respectively by brief treatment with aziridine in refluxing ethanol (Scheme 1). The presence of a tertiary amine base was necessary to inhibit the acid-catalysed polymerisation known to occur for the unlabelled analogue. Careful monitoring of the progress of the addition reaction by TLC was also needed for optimum yield. The labelled epoxides reacted smoothly with excess piperidine giving the required isotopomers of pimonidazole (9a,b), again in very high yields.

The use of the above syntheses allowed [^{3}H]-RSU 1069 and [^{3}H]-pimonidazole to be prepared in sufficiently high specific activity for use in experiments designed to examine the selectivity of covalent binding with DNA and other macromolecules of cells *in vitro* in hypoxia and in the presence of oxygen. Similarly, assessment of the extent and selectivity of retention of these radiosensitisers and bioreductively-activated cytotoxins in tumours and in various normal tissues by autoradiography is facilitated. The results of these biological studies will be reported elsewhere.

EXPERIMENTAL

Solutions were dried over anhydrous sodium sulphate and were filtered prior to evaporation of the solvents under reduced pressure. Melting points were uncorrected. NMR spectra were obtained using a Jeol PMX60SI spectrometer with tetramethylsilane as internal standard. IR spectra were recorded on Nujol mulls using a Philips PU9510 instrument. Determination of radioactivity was carried out by liquid scintillation counting using Beckman LS2800 and Beckman LS5000CE instruments. Radiochemical purities were estimated by scintillation counting of appropriate portions of silica scraped from TLC analyses. Sodium boro[^{3}H]hydride was obtained from Aldrich Chemical Co. and sodium boro[^{3}H]hydride was obtained from Amersham International PLC.

1-(3-Chloro-2-oxopropyl)-2-nitroimidazole (5).- 1-(3-Chloro-2-hydroxy-propyl)-2-nitroimidazole (4; 2.06 g, 10 mmol) (prepared from 2-nitroimidazole and 3-chloro-1,2-epoxypropane by the method of Beaman et al (16)) was stirred with chromium trioxide (1.0 g, 10 mmol) and sulphuric acid (2 ml) in acetone (40 ml) for 3 days. Sodium hydrogen carbonate (8 g) was added and the mixture was filtered. The solvent was evaporated from the filtrate and the residue, in ethyl acetate, was washed with water and with saturated aqueous sodium hydrogen carbonate before being dried. The evaporation residue was recrystallised from ethyl acetate to afford 5 (936 mg, 46 %) as white crystals m.p. 93-94°C; ν max 1750 cm^{-1}; δ ($CDCl_3$: $(CD_3)_2SO$, 3:1) 4.48 (2 H, s, CH_2Cl), 5.77 (2 H, s, imidazole-CH_2), 7.12 (1 H, brs, imidazole-H), 7.35 (1 H, brs, imidazole-H).

1-(2,3-Epoxy-2-[^{2}H]propyl)-2-nitroimidazole (7a).- The chloromethylketone 5 (203 mg, 1.0 mmol) was stirred with NaB^2H_4 (98 atom %; 12.2 mg, 0.29 mmol) in absolute ethanol (15 ml) for 20 min. Acetone (3 ml) was then added and the mixture was stirred for 1 h before evaporation of the solvents. The residue was stirred vigorously with aqueous sodium hydroxide (10 %, 2.0 ml, 5.0 mmol) for 30 min. The suspension was diluted with water (5 ml) and was extracted with chloroform (3 x 15 ml). The combined extracts were dried and the solvent was evaporated to give 7a (130 mg, 76 %) as an off-white crystalline solid m.p. 53-55°C; δ ($CDCl_3$) 2.55 (1 H, d, J = 4.5 Hz) and 2.90 (1 H, d, J = 4.5 Hz, epoxide-CH_2), 4.25 (1 H, d, J = 14.5 Hz) and 4.95 (1 H, d, J = 14.5 Hz) NCH_2, 7.08 (1 H, s, imidazole-H), 7.20 (1 H, s, imidazole-H).

Experiment to determine k[^{1}H]/k[^{2}H] for the reduction of 5.- A solution of NaB^1H_4 (37.5 mg, 0.987 mmol) and NaB^2H_4 (98 atom %; 410 mg, 0.976 mmol) in ethanol (25 ml) was added to ketone 5 (407 mg, 2.0 mmol) and the resulting solution was stirred for 15 min. Acetone (5 ml) was added and the mixture was stirred for 16 h before evaporation of the solvents. The residue was treated with aqueous sodium hydroxide as above to afford a pale yellow crystalline solid (320 mg, 94 %) which was shown by NMR to comprise a mixture of isotopomers of 1-(2,3-epoxypropyl)-2-nitroimidazole. Integration of the multiplet centred at

δ 3.44 showed that the mixture contained the [^{1}H]-epoxide (61 %) and the [^{2}H]-epoxide (39 %). From these data it was calculated that $k[^1H]/k[^2H]$ = 1.5 for the reduction step.

1-(2,3-Epoxy-2-[^{3}H]propyl)-2-nitroimidazole (7b).- Sodium borohydride (1 mg, 0.026 mmol) was added to ketone 5 (203 mg, 1.0 mmol) in ethanol (10 ml), followed after 5 min by sodium boro[^{3}H]hydride (1.8 mg; 0.048 mmol, 220 mCi) in ethanol (1 ml) and water (0.5 ml). After a further 20 min, sodium borohydride (40 mg, 1.05 mmol) was added and the mixture was stirred for 30 min. Acetone (3 ml) was then added and the mixture was stirred for 16 h before evaporation of the solvents. The residue was stirred vigorously with 10 % aqueous sodium hydroxide (4.0 ml, 10 mmol) for 35 min. The suspension was diluted with water (10 ml) and was extracted with chloroform (3 x 50 ml). The combined extracts were dried and the solvent was evaporated to give 7b (160 mg, 95 % chemical yield; 189 mCi, 86 % radiochemical yield) which co-chromatographed (TLC, silica, $CHCl_3$/MeOH, 9:1, Rf 0.6) with authentic unlabelled material. The radiochemical purity was > 96 % and the specific activity was 200 mCi mmol^{-1}.

1(3-(Aziridin-1-yl)-2-hydroxy-2-[^{2}H]propyl)-2-nitroimidazole (8a).- The [^{2}H]-epoxide 7a (120 mg, 0.7 mmol) was boiled under reflux in ethanol (2 ml) with aziridine (0.09 ml; 75 mg, 1.7 mmol) and triethylamine (0.03 ml) for 15 min. Evaporation of the solvents and excess reagent gave a solid which was triturated with cold acetone to afford 8a (140 mg, 94 %) as a pale yellow solid m.p. 116-118°C; δ (CD_3OD) 1.4 (2 H, m) and 1.8 (2 H, m) aziridine-H, 2.25 (1 H, d, J = 12 Hz) and 2.45 (1 H, d, J = 12 Hz) aziridine-C$\underline{H}_2$CD(OH)-, 4.30 (1 H, d, J = 14 Hz) and 4.68 (1 H, d, J = 14 Hz) imidazole-C$\underline{H}_2$CD(OH)-, 4.75 (1 H, s, OH), 7.03 (1 H, d, J = 2 Hz, imidazole-H), 7.37 (1 H, d, J = 2 Hz, imidazole-H).

1-(3-(Aziridin-1-yl)-2-hydroxy-2-[^{3}H]propyl)-2-nitroimidazole (8b).- Epoxide 7b (100 mg, 0.59 mmol; 118 mCi) was boiled under reflux in ethanol (5 ml) with aziridine (0.35 ml) and triethylamine (0.1 ml) for 20 min. Evaporation of the

solvents and excess reagent gave 8b (125 mg, quant.; 118 mCi, quant.) which co-chromatographed (TLC, silica, $CHCl_3$/MeOH, 9:1, Rf 0.15) with authentic unlabelled material. The radiochemical purity was > 96 % and the specific activity was 200 mCi mmol^{-1}.

1-(2-Hydroxy-3-(piperidin-1-yl)-2-[^{2}H]propyl)-2-nitroimidazole (9a).- The [^{2}H]-epoxide 7a (240 mg, 1.4 mmol) was boiled under reflux in ethanol (20 ml) with piperidine (260 mg, 3.1 mmol) for 15 min. Evaporation of the solvent and excess reagent gave 9a (360 mg, 99 %) as pale yellow crystals m.p. 107-108°C; ν max (Nujol mull) 3320 br, 2160 cm^{-1}; δ ($CDCl_3$) 1.5 (6 H, br, piperidine 3,4,5-CH_2), 2.1-2.8 (6 H, m, $CDCH_2N$ + piperidine 2,6-CH_2), 4.22 (1 H, d, J = 14 Hz) and 4.64 (1 H, d, J = 14 Hz) imidazole-$CH_2CD(OH)$-, 4.28 (1 H, s, OH), 6.98 (1 H, brs, imidazole-H), 7.25 (1 H, brs, imidazole-H).

1-(2-Hydroxy-3-(piperidin-1-yl)-2-[^{3}H]propyl)-2-nitroimidazole (9b).- Epoxide 7b (30 mg, 0.18 mmol; 35 mCi) was boiled under reflux in ethanol (1.5 ml) with piperidine (15 mg, 0.534 mmol) for 5 min. Evaporation of the solvents and excess reagent gave 9b (45 mg, quant.; 35 mCi, quant.) which co-chromatographed (TLC, silica, $CHCl_3$/MeOH, 9:1, Rf 0.2) with authentic unlabelled material. The radiochemical purity was > 96 % and the specific activity was 200 mCi mmol^{-1}.

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PUBLICATION 37

Labelled Compounds of Interest as Antitumour Agents. Part III.
Synthesis of ^{2}H and ^{3}H Isotopomers of Etanidazole (SR 2508)

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LABELLED COMPOUNDS OF INTEREST AS ANTITUMOUR AGENTS. PART III (1). SYNTHESIS OF ^{2}H AND ^{3}H ISOTOPOMERS OF ETANIDAZOLE (SR 2508).

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SUMMARY

Chemically and radiochemically efficient syntheses of N-(2-hydroxy-2-[^{2}H]ethyl)-2-(2-nitroimidazol-1-yl)acetamide and N-(2-hydroxy-2-[^{3}H]ethyl)-2-(2-nitroimidazol-1-yl)acetamide, isotopomers of the hypoxic cell radiosensitiser etanidazole (SR 2508), have been achieved by reduction of the corresponding aldehyde with isotopically labelled sodium borohydride. The primary kinetic deuterium isotope effect for the process is 1.5.

Key words: [^{2}H]-Etanidazole, [^{3}H]-Etanidazole, Etanidazole, SR 2508.

INTRODUCTION

Etanidazole (SR 2508; 1; Figure 1) (2,3) is a member of the second generation of electron-affinic compounds based on 2-nitroimidazole, designed for the sensitisation of hypoxic tumour cells to radiation. Particularly, it elicits less neurotoxicity than the archetype misonidazole (2). It is also significantly more polar than the latter. It was therefore of interest to assess the selectivity of uptake and covalent retention of 1 in hypoxic and oxic regions of tumours and in normal tissues, including brain. Radiolabelled material was required for this purpose .

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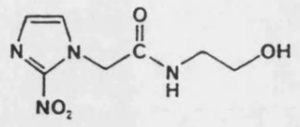

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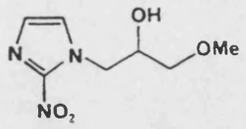

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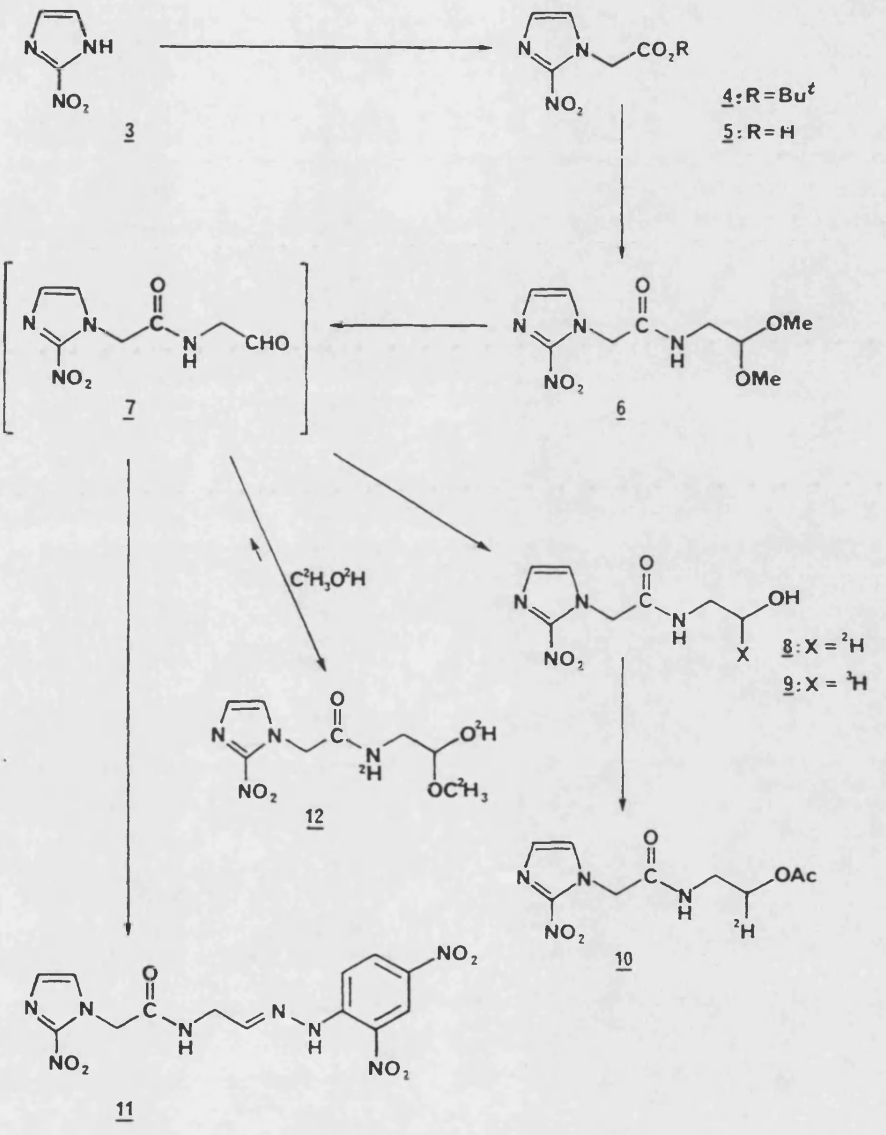
FIGURE 1.

We have reported (1) the efficient and rapid incorporation of ^{2}H and ^{3}H by the reduction of a doubly activated ketone by the appropriate isotopically labelled sodium borohydride. It was therefore considered highly likely that the analogous reduction of the activated aldehyde 7 would be successful in providing the required isotopically labelled materials.

Synthesis of 7 was achieved in four steps as shown in Scheme 1. 2-Nitroimidazole (3) was alkylated with t-butyl bromoacetate under basic conditions in refluxing dimethylformamide and the imidazoleacetic acid 5 was formed by treatment of the intermediate t-butyl ester 4 with anhydrous trifluoroacetic acid. This route was found to avoid many of the previously reported problems (4) of isolation and purification associated with the great aqueous solubility and acid strength of 5. The acid chloride of 5 proved to be difficult to form and to be unstable, so a carbodiimide method was used to couple this acid with 2,2-dimethoxyethylamine, albeit in modest yield. Hydrolysis of the acetal function of 6 was achieved using an ion-exchange resin as catalyst. The resultant aldehyde 7 was found to be highly electrophilic, being extensively hydrated in water and forming fully the corresponding hemiacetal 12 when an attempt was made to record the ^{1}H NMR spectrum in solution in [$^{2}H_{4}$]methanol. The dinitrophenylhydrazone 11 was prepared for characterisation.

For the purpose of the reductions with sodium boro[^{2}H]hydride, 7 was not isolated but was used as the neutral aqueous solution given by removal of the catalytic resin by filtration; the [^{2}H]-primary alcohol 8 was obtained in good yield. To determine the kinetic isotope effect for this reduction step, the aqueous solution of 7 was reduced with an excess of an equimolar mixture of



SCHEME 1.

NaB^1H_4 and NaB^2H_4. Analysis of the resulting mixture of isotopomers by NMR was facilitated by acetylation, thus removing the overlapping of the $NC^1\underline{H}_2C^1H^2HOH$ and $NC^1H_2C^1\underline{H}^2HOH$ resonances in the spectrum of 8. Comparison of the integrals of signals at δ 3.45 ($CONC^1H_2$), and δ 4.10 ($C^1\underline{H}^2HOAc$ + $C^1\underline{H}_2OAc$) enabled the estimation $k[^1H]/k[^2H] = 1.5$ for the reduction step. To avoid possible poor incorporation of ^3H from sodium boro[^3H]hydride through unfavourable competition

with ^1H, the 'cold chaser' technique (1) was used and the radiochemical yield of 9 was excellent. Interestingly, Coleman et al (5) report that the corresponding unlabelled acetate ester is a good prodrug for 1.

The development of this facile and high-yielding preparation of radiolabelled etanidazole permits studies of selectivity in uptake and retention of the drug and its metabolites in normal tissues and in tumours. The results of these experiments will be published elsewhere.

EXPERIMENTAL

DMF refers to dimethylformamide. Tetrahydrofuran (THF) was distilled from calcium hydride before use. Solutions were dried with anhydrous sodium sulphate and were filtered prior to evaporation of the solvents under reduced pressure. Melting points are uncorrected. NMR spectra were obtained using a Jeol PMX60SI spectrometer with tetramethylsilane as internal standard. IR spectra were recorded using a Philips PU9510 instrument. Determinations of the radioactivity were carried out by the liquid scintillation method using Beckman LS2800 and Beckman LS5000CE instruments. The radiochemical purity of 9 was checked by scraping appropriate portions of an analytical thin layer chromatography plate (silica gel: $CHCl_3$: MeOH, 4:1) and assaying radioactivity by liquid scintillation counting. Sodium boro[^2H]hydride was obtained from Aldrich Chemical Co. and sodium boro[^3H]hydride was obtained from Amersham International PLC.

t-Butyl 2-(2-nitroimidazol-1-yl)acetate (4).- 2-Nitroimidazole (3; 4.57 g, 40.4 mmol) was stirred at reflux in DMF (40 ml) with potassium t-butoxide (4.85 g, 43.3 mmol) for 5 min before being cooled to 120°C. t-Butyl bromoacetate (7.90 g, 40.5 mmol) was added and the whole was stirred at reflux for a further 5 min. The cooled mixture, in ethanol (100 ml) was filtered and the solvents were evaporated at 1 torr. The residue, in dichloromethane, was washed with water (4 x) and saturated aqueous sodium chloride before being dried. The

solvent was evaporated to give 4 (6.38 g, 70 %) as a pale yellow oil; δ ($CDCl_3$) 1.45 (9 H, s, $C(CH_3)_3$), 5.02 (2 H, s, CH_2), 7.1 (2 H, br, 2 x imidazole-H).

2-(2-Nitroimidazol-1-yl)acetic acid (5).- The t-butyl ester 4 (6.29 g, 27.7 mmol) was stirred with trifluoroacetic acid (40 ml) for 16 h before evaporation of the reagent. The residual solid was washed with a mixture of diethyl ether and hexane (1:1) to afford 5 (4.74 g, quant.) as a pale yellow solid m.p. 145-146°C (lit. (4) m.p. 159-160°C (expl.)); δ ($CDCl_3$) 5.20 (2 H, s, CH_2), 7.52 (1 H, d, J = 1.5 Hz, imidazole-H), 7.58 (1 H, d, J = 1.5 Hz, imidazole-H).

N-(2,2-Dimethoxyethyl)-2-(2-nitroimidazol-1-yl)acetamide (6).- The carboxylic acid 5 (510 mg, 3 mmol) was stirred with N,N'-dicyclohexylcarbodiimide (630 mg, 3 mmol) and 2,2-dimethoxyethylamine (360 mg, 3 mmol) in THF (13 ml) for 3 days before the mixture was filtered and the solvent was evaporated. Centrifugally-accelerated preparative layer chromatography (silica gel, ethyl acetate:hexane, 1:1, followed by ethyl acetate) gave a crude solid which was recrystallised from ethyl acetate:hexane to afford 6 as an off-white solid m.p. 116-117°C; δ ($CDCl_3$:$(CD_3)_2CO$, 1:2) 3.30 (6 H, s, 2 x OCH_3), 3.35 (2 H, t, J = 5 Hz, NCH_2CH), 4.35 (1 H, t, J = 5 Hz, NCH_2CH), 5.15 (2 H, s, imidazole-CH_2), 7.07 (1 H, d, J = 1.5 Hz, imidazole-H), 7.17 (1 H, d, J = 1.5 Hz, imidazole-H), 7.55 (1 H, br, NH).

N-(2-Hydroxy-2-[^{2}H]ethyl)-2-(2-nitroimidazol-1-yl)acetamide (8) and N-(2-Acetoxy-2-[^{2}H]ethyl)-2-(2-nitroimidazol-1-yl)acetamide (10).- The acetal 6 (258 mg, 1 mmol) was boiled under reflux with Dowex 50X4 (H^+ form; 300 mg) in THF (5 ml) and water (3 ml) for 1 h before the suspension was filtered to give a solution of the aldehyde 7. Sodium boro[^{2}H]hydride (NaB^2H_4; 42 mg, 1 mmol), in water (2 ml) was added to the cooled filtrate and the mixture was stirred for 5 min before addition of acetone (3 ml). After a further 16 h, the solvents were evaporated and the residue, in water (5 ml), was applied to a short column of Amberlite IRA-400 (^-OH form; 1.0 g) and was eluted with a water (10 ml). This

eluate was then passed through a short column of Dowex 50X4 (H^+ form; 1.0 g) and the water was evaporated from the final eluate to give 8 (160 mg, 74 %) as an off-white solid m.p. 133-136°C; ν max (Nujol mull) 3400, 3300, 2120 , 1665 cm^{-1}; δ ($CDCl_3$ + $(CD_3)_2SO$; 1:1) 3.2-3.6 (3 H, m, NCH_2CHD), 5.15 (3 H, brs, imidazole-CH_2 + OH), 7.10 (1 H, brs, imidazole-H), 7.45 (1 H, br imidazole-H) and 8.20 (1 H, brt, J = 6 Hz, NH). A small sample was treated with acetic anhydride and pyridine to afford 10 as a pale yellow solid 2.02 (3 H, s, $COCH_3$), 3.45 (2 H, t, J = 7 Hz (becomes d, J = 7 Hz on decoupling at δ 8.32), $CONCH_2$), 4.10 (1 H, brt, J = 7 Hz, CHD), 5.15 (2 H, s, imidazole-CH_2), 7.13 (1 H, brs, imidazole-H), 7.43 (1 H, brs, imidazole-H) and 8.30 (1 H, brt, J = 6 Hz, NH).

N-(2-Hydroxy-2-[^{3}H]ethyl)-2-(2-nitroimidazol-1-yl)acetamide (9).- Acetal 6 (136 mg, 0.51 mmol) was hydrolysed with Dowex 50X4 in aqueous THF and the resin was removed by filtration as described above. Sodium borohydride (1 mg, 0.026 mmol) was added, followed after 5 min by an aqueous solution of sodium boro[^{3}H]hydride (0.0005 mmol, 2.5 mCi; 0.5 ml). After a further 5 min, sodium borohydride (19 mg, 0.5 mmol) was added and the mixture was stirred for 15 min before addition of acetone (2 ml). After a further 16 h, the solvents were evaporated and the residue, in water (5 ml), was applied to a short column of Amberlite IRA-400 (^-OH form; 0.7 g) and was eluted with a water (7 ml). This eluate was then passed through a short column of Dowex 50X4 (H^+ form; 1.0 g) and the water was evaporated from the final eluate to give 9 (95 mg, 2.0 mCi, chemical yield 87 %, radiochemical yield 80 %) with TLC properties identical to those of an authentic sample of 1 (silica gel, $CHCl_3$:MeOH, 4:1, Rf 0.2). The radiochemical purity was > 97 % and the specific activity was 4.5 mCi $mmol^{-1}$.

N-(2-Oxoethyl)-2-(2-nitroimidazol-1-yl)acetamide 2,4-Dinitrophenylhydrazone (11).- A solution of the aldehyde 7 (0.7 mmol) was prepared by hydrolysis of 6 with Dowex 50X4 in aqueous THF as described above. Treatment with 2,4-dinitrophenylhydrazine (150 mg, 0.75 mmol) in ethanol for 5 min afforded, after recrystallisation from water, 11 as bright yellow-orange crystals m.p. 239-242°C; δ ($(CD_3)_2SO$) 4.05 (2 H, dd, J = 5 Hz, J = 3.5 Hz (becomes d, J = 5 Hz on

decoupling at δ 8.00; becomes d, J = 3.5 Hz on decoupling at δ 8.75), NCH_2CHN), 5.25 (2 H, s, imidazole-CH_2), 5.7 (1 H, br, ArNH), 7.25 (1 H, d, J = 1 Hz, imidazole-H), 7.70 (1 H, d, J = 1 Hz, imidazole-H), 7.95 (1 H, d, J = 9 Hz, Ar 6-H), 8.00 (1 H, m (becomes s on decoupling at δ 4.05), NCH_2CHN), 8.40 (1 H, dd, J = 9 Hz, J = 2 Hz, Ar 5-H), 8.75 (1 H, t, J = 5 Hz (becomes s on decoupling at δ 4.05), $CONHCH_2$), 8.85 (1 H, d, J = 2 Hz, Ar 3-H).

Experiment to determine k[^{1}H]/k[^{2}H] for the reduction of 7.- Acetal 6 was hydrolysed with Dowex 50X4 in aqueous THF, reduced and treated with acetic anhydride in pyridine as above, except that the reductant was an excess of an equimolar mixture of NaB^1H_4 and NaB^2H_4 (98 atom %). The residue was shown by NMR to comprise a mixture of isotopomers of N-(2-acetoxyethyl)-2-(2-nitroimidazol-1-yl)acetamide. Integration of the triplet centred at δ 4.10 showed that the mixture contained 65 % of the ^{1}H-compound and 35 % of the ^{2}H-compound. From these data it was calculated that k[^{1}H]/k[^{2}H] = 1.5 for the reduction step.

ACKNOWLEDGEMENT.

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PUBLICATION 38

Synthesis and Evaluation of Novel Electrophilic Nitrofuran Carboxamides and Carboxylates as Radiosensitizers and Bioreductively-Activated Cytotoxins

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Synthesis and Evaluation of Novel Electrophilic Nitrofuran Carboxamides and Carboxylates as Radiosensitizers and Bioreductively Activated Cytotoxins

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A series of 5-nitrofuran-2- and 3-carboxamides bearing alkylating side-chains has been synthesized and tested for their ability to radiosensitize selectively hypoxic Chinese hamster cells (V79) to the lethal effects of ionizing radiation and also for their ability to act directly and selectively as cytotoxic drugs on hypoxic V79 cells. The compounds were extremely efficient radiosensitizers of such cells in vitro and were more efficient than known nitroimidazoles of similar type. Their efficiencies as radiosensitizers correlated with their high electron affinity (E_7^1) as measured by pulse-radiolysis. However the compounds showed little radiosensitizing activity towards KHT sarcomas in C3H mice. The compounds in this series of nitrofurans were generally more toxic towards hypoxic cells than towards oxic cells in vitro but were less effective upon the basis of a differential effect than were similar nitroimidazoles reported previously.

Nitroheterocyclic compounds can act as radiosensitizers of hypoxic cells and as bioreductively activated cytotoxins.[1] Radiosensitization is a fast, free-radical process, and a correlation has been observed between the one-electron reduction potentials (E_7^1) of a large number of chemically diverse nitroheterocycles and their ability to act as radiosensitizers of hypoxic cells.[2,3] Nitro compounds can be reductively metabolized to form highly potent cytotoxins. Since bioreductive activation occurs more readily in hypoxic tissue, there is a sound basis for a high degree of specificity in poorly oxygenated solid tumors.

Both the bioreductive activity and radiosensitizing efficiency of 2-nitroimidazoles can be greatly increased by incorporating monofunctional alkylating groups into the molecule. One of the first examples of such a compound was α-(1-aziridinylmethyl)-2-nitro-1*H*-imidazole-1-ethanol (RSU-1069, 1),[4,5] a 2-nitroimidazole bearing an aziridine (Figure 1). This compound is about 5–10-fold more efficient than misonidazole (a related nitroimidazole not containing an alkylating group) as a radiation sensitizer of experimental tumors and shows considerably more cytotoxicity when reductively metabolized under hypoxic conditions. Preliminary clinical investigation of RSU-1069 has revealed gastrointestinal toxicity which restricts doses to levels not likely to produce significant radiosensitization.[6] Various analogues have been synthesized and evaluated in attempts to reduce toxicity towards normal tissues without a corresponding reduction in radiosensitization of tumors. Examples include compounds in which the aziridine group is deactivated by substitution.[7] Lower toxicity can also be achieved by using a prodrug of RSU-1069 such as the new and recently reported compound α-[[(2-bromoethyl)amino]methyl]-2-nitro-1*H*-imidazole-1-ethanol hydrobromide (RB-6145, 2).[8]

Increasing therapeutic benefit can also be achieved by increasing radiosensitizing potency. One possible way of obtaining this is to raise the redox potential of the nitroheterocycle. For example 5-nitrofurans are generally more electron affinic than 2-nitroimidazoles and both 5-nitro-2-furaldehyde semicarbazone (nitrofurazone, 3) and 5-nitro-2-furaldoxime (nifuroxime, 4) have been found to be very effective radiosensitizers of hypoxic cells in tissue culture systems.[9,10] We have investigated nitrofurans bearing alkylating side-chains, and with E_7^1 values of between −210 and −350 mV (ie; both higher and lower E_7^1 than the known nonalkylating sensitizer nitrofurazone, 3) by altering the patterns of substitution. Of particular importance is the position of the nitro group on the furan ring and possible conjugation with electron withdrawing substituents. Changes in the nature of the substituents likely to have an effect on electron affinity have also been investigated. This paper describes studies with the nitrofurans having the highest (most positive) electron affinities of those synthesized, namely the 5-nitrofuran carboxamides and propenamides bearing side chains of varying electrophilic reactivity.

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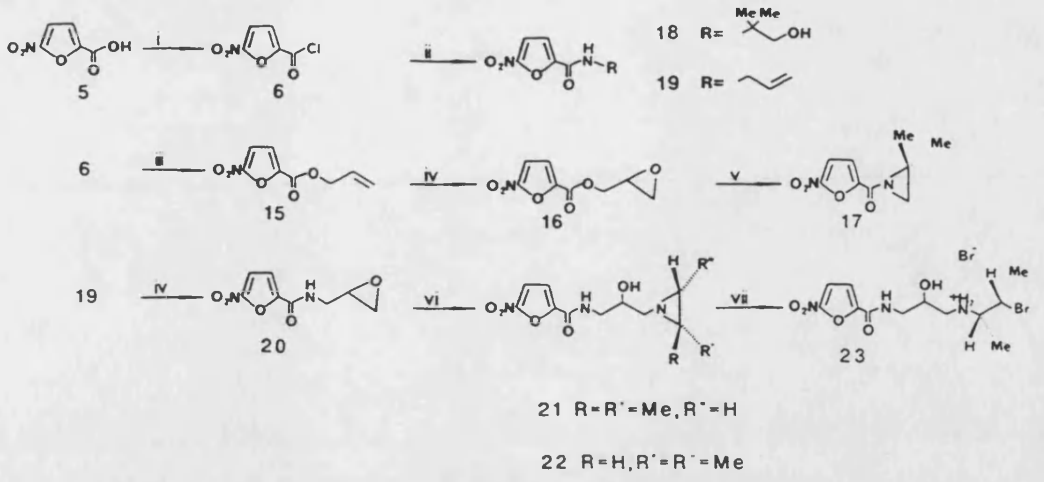
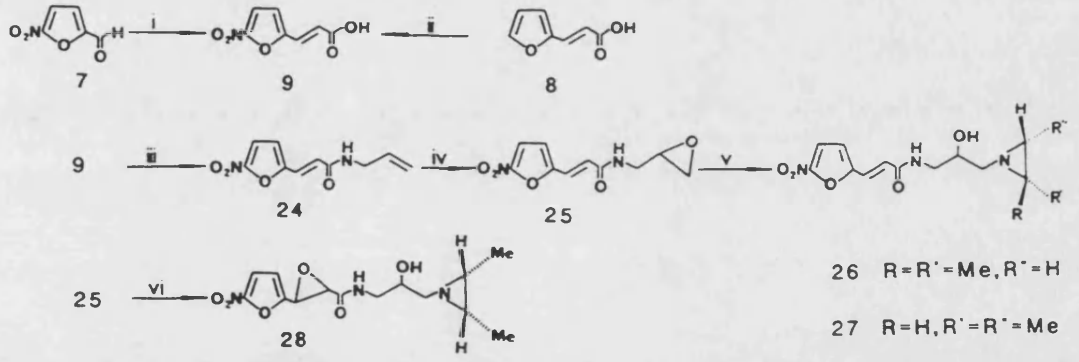
Scheme I[a]



[a] Reagents: (i) $SOCl_2/DMF$; (ii) $RNH_2/Et_3N/CH_2Cl_2$; (iii) allylalcohol/Et_3N/CH_2Cl_2; (iv) $MCPBA/CH_2Cl_2$; (v) 2,2-dimethylaziridine/THF; (vi) *cis*-2,3-dimethylaziridine/THF; (vii) HBr (aq).

Scheme II[a]



[a] Reagents: (i) $Ac_2O/NaOAc$; (ii) Ac_2O/HNO_3; (iii) allylamine/Et_3N/CH_2Cl_2; (iv) $MCPBA/CH_2Cl_2$; (v) 2,2-dimethylaziridine/THF; (vi) *cis*-2,3-dimethylaziridine/THF/MCPBA/Et_3N.

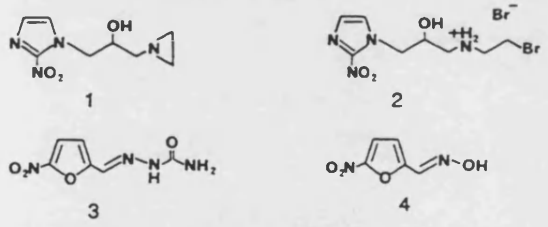


Figure 1.

Chemistry

The common starting material for the series of 5-nitrofuran-2-carboxamides **15–23** (of which, **15** has been reported previously[11]) was 5-nitro-2-furoyl chloride, prepared from commercially available 5-nitro-2-furoic acid (**5**) by treatment with refluxing thionyl chloride in the presence of a catalytic amount of dimethylformamide (DMF). Subsequent amides or esters were then prepared directly from this intermediate with the appropriate alcohol or amine (Scheme I).

Compounds **24–28** were prepared from the common starting material 5-nitro-2-furylacrylic acid (**9**), prepared by Perkin condensation from 5-nitro-2-furaldehyde (**7**) with acetic anhydride/sodium acetate or directly by nitration of 2-furylacrylic acid (**8**). Corresponding propenamides (of which, **24** has been reported previously as an antibacterial agent[12]) were then prepared from the acyl chloride (Scheme II).

Compounds **29** and **30** were prepared by nitration of 2-methyl-3-furoic acid (**11**) according to the procedure of Gilman et al.,[13] followed by coupling to allylamine by a mixed anhydride method. This coupling was used owing to the instability of 2-methyl-5-nitrofuran-3-carboxylic acid (**12**) in the presence of thionyl chloride. The 3-nitro analogue **31** was prepared from 2-methyl-5-furoic acid by nitration of the methyl ester (**13**) according to the method of Rinkes,[14] followed by direct coupling of the hydrolyzed ester (**14**) with allylamine (Scheme III).

Oxiranes **16**, **20**, **25**, and **30** were generally synthesized from the allyl amide or ester by treatment with 3-chloroperbenzoic acid (MCPBA). With the exception of **30**, which was broken down to unidentified products in refluxing aziridines under a variety of conditions, treatment of these oxiranes with the appropriate aziridines in boiling THF gave the N-substituted aziridines (**21**, **22**, **26**, and **27**,

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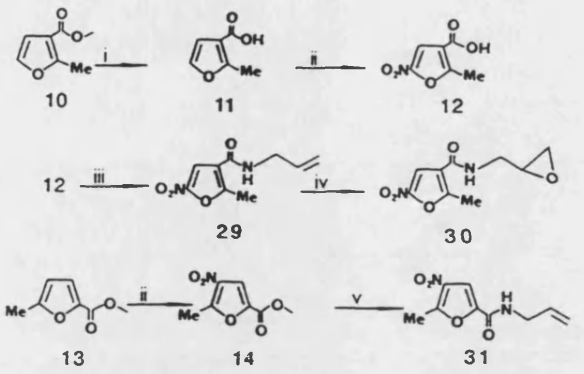
Scheme III[a]



[a] Reagents: (i) $NaOH/H_2O/EtOH$; (ii) Ac_2O/HNO_3; (iii) ethyl chloroformate/allylamine/Et_3N/CH_2Cl_2; (iv) $MCPBA/CH_2Cl_2$; (v) NaOH/EtOH then allylamine/DCC/THF.

Schemes I and II). Treatment of the oxiranes with unsubstituted aziridine was unsuccessful due to the instability of the products. Treatment of the ester **16** with 2,2-dimethylaziridine gave only the *N*-acyl aziridine **17** by reaction at the carbonyl center of the molecule rather than at the oxirane (Scheme I). 2,2-Dimethylaziridine and stereochemically pure *cis*-2,3-dimethylaziridine were prepared from the appropriately substituted 2-aminoethanols by O-sulfation and elimination with potassium hydroxide, according to the methods of Dickey et al.[15] The potentially bifunctionally alkylating analogue **28** was prepared by reaction of **25** with *cis*-2,3-dimethylaziridine in the presence of MCPBA and triethylamine (Et_3N). Compound **18**, with a nonalkylating hydroxyethyl substituent was prepared from the corresponding acyl chloride and the appropriate amine as described. The ring-opened analogue **23** was prepared by treatment of **21** with HBr by methods similar to those we have described elsewhere for a series of 2-nitroimidazoles.[8]

Results and Discussion

The 5-nitrofurans investigated show high radiosensitizing activity in vitro with $C_{1.6}$ values of as low as 8 μM for compounds **25** and **26**, 5-nitro-2-furanpropenamides with oxiranyl and 2,2-dimethylaziridinyl substituents, respectively (Table I). Generally $C_{1.6}$ values of 10–50 μM were found for this series of nitrofurans. These radiosensitizing efficiencies compare favorably with that of compound **3** and all compounds are more efficient radiosensitizers than the nitroimidazoles such as misonidazole and RSU-1069 (**1**),[4,5] as would be expected for such highly electron affinic compounds. The less oxidizing 3-nitro analogue, **31**, is considerably less effective as a radiosensitizer. With the exception of compounds **25** and **26**, which were considerably more potent sensitizers than their nonalkylating analogues, there appears to be little benefit in terms of radiosensitizing efficiency when bifunctional character is introduced in this series of compounds. This is in contrast to the trends observed with nitroimidazoles.

The 5-nitrofurans in this study showed some selective activity in the assay used for direct bioreductive cytotoxicity. The highest ratios of air to nitrogen toxicity (C_{50}(air)/C_{50}(N_2)) are displayed by those compounds having aziridinyl or (bromoethyl)amino substituents, but these ratios are lower than those observed in our previous studies with nitroimidazoles. For example, compounds bearing the 2,2-dimethylaziridine substituent (**21** and **26**) are 5–10 times less effective as hypoxia-selective cytotoxins than is the corresponding nitroimidazole analogue α-((2,2-dimethyl-1-aziridinyl)methyl)-2-nitro-1*H*-imidazole-1-ethanol (RSU-1150).[7] The most potent compound showing direct hypoxia-selective cytotoxic properties is the (2-bromo-1,1-dimethylethyl)amino compound **23**, with a differential toxicity ratio of 6. This is a comparable differential to that found for the known nitrofuran, nitrofurazone **3**, but the oxic and hypoxic toxicities are 10 times higher. With the exception of **25**, differential cytotoxicity was not shown by the corresponding oxiranes.

Table I. Physicochemical and in Vitro Biological Data of Nitrofurans and Selected Nitroimidazoles

compound	$-E_7^1$, mV	radiosensitization:[a] $C_{1.6}$, mM	C_{50},[b] mM air	C_{50},[b] mM N_2	d tox[c]
misonidazole	389[d]	0.3[d]	45.0	4.0	11.3
1	389[e]	0.1[f]	0.3	0.0045	66.6
2	nd[m]	0.1[g]	2.3[h]	0.09[h]	25.6[h]
3	257[i]	0.05[j]	1.7	0.2	8.5
15	211[k]	0.05	0.1	0.025	4.0
16	268[k]	0.025	0.2	0.075	2.7
17	nd	0.1	0.18	0.18	1.0
18	220[k]	0.05	0.25	0.12	2.1
19	nd	0.05	0.08	0.045	1.8
20	241[k]	0.02	0.05	0.05	1.0
21	nd	0.04	0.15	0.075	2.0
22	219[k]	0.05	0.06	0.02	3.0
23	nd	0.03	0.14	0.025	5.6
24	nd	0.03	0.06	0.025	2.4
25	252[k]	0.008	0.03	0.01	3.0
26	nd	0.008	0.045	0.01	4.5
27	231[k]	0.035	0.07	0.04	1.8
28	335[k]	0.025	0.012	0.012	1.0
29	325[k]	0.075	0.35	0.8	0.4
30	327[k]	0.04	0.1	0.1	1.0
31	>600[k]	nd[l]	0.015	0.03	0.5

[a] $C_{1.6}$ = molar concentration of the compound required to give a sensitizer enhancement ratio (ER) of 1.6. Hence the lower the value of $C_{1.6}$ the greater is the sensitizing efficiency. [b] C_{50} (Air or N_2) = concentration required to reduce optical density by 50% in the MTT assay (proportional to cell killing).[20] [c] d tox = differential toxicity; C_{50} (air)/C_{50} (N_2) (mean of at least three experiments). [d] Data taken from ref 3. [e] Data taken from ref 21. [f] Data taken from ref 7. [g] Data taken from ref 8. [h] These values are concentrations required to reduce cell survival to 1%.[8] [i] Data taken from ref 22. [j] Data taken from ref 2. [k] Referred to the normal hydrogen electrode (NHE) using benzyl viologen as redox couple; $[BV^{2+}/BV^{\cdot+}]$ = −354 mV. [l] Not determined due to toxicity. ER = 1.4 at 0.02 mM. [m] nd = not determined.

The toxicities of these compounds towards oxic cells are at least 1 order of magnitude greater than are the toxicities of similar nitroimidazoles, a property which could account for the poor differentials observed. High oxic toxicities could be related to high one-electron reduction potentials,[2] coupled with alkylating moieties. However, a number of compounds (**15**, **18**, **19**, and **24**) have higher toxicities than **3** despite comparable electron-affinities and similarly do not possess alkylating moieties. It therefore appears that other factors are contributing towards toxicity in this series of furans. One-electron reduction potentials (E_7^1) measured at pH 7 for a selection of the 5-nitrofuran-2-carboxamides and propenamides using a pulse-radiolytic method were found to be between −210 and −270 mV, ie; 100–150 mV more positive than the nitroimidazoles (Table I). There is some variation in E_7^1 with changes in the side chain at the 2-position. The compounds bearing aziridinyl groups are more electron affinic than their oxiranyl analogues, but sensitizing efficiency was unaffected by these changes. In view of the high oxic toxicity and poor hypoxia selectivity that appears to be related to highly positive E_7^1, the re-

(15) Dickey, F. H.; Fickett, W.; Lucas, H. J. *J. Am. Chem. Soc.* 1952, *74*, 944.

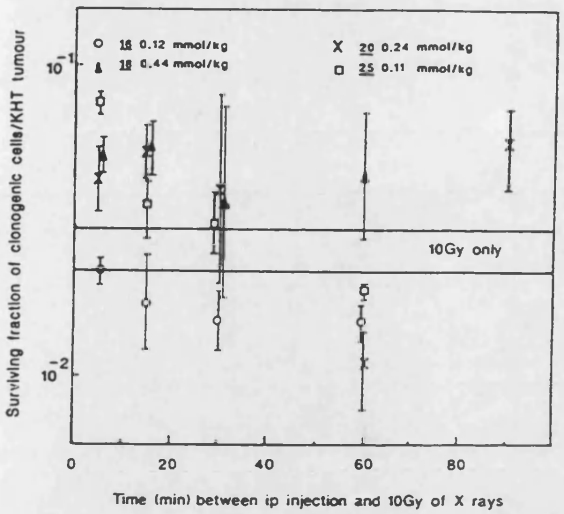


Figure 2. Evaluation of nitrofurans as radiosensitizers in vivo.

duction potential was lowered to below −300 mV by the synthesis of analogues bearing the functionalized side chain at the 3-position and a methyl group at the 2-position (compounds **29** and **30**). These analogues were less toxic than the corresponding 2-carboxamides but showed no improvement in differential toxicity. Compound **28**, in which the conjugation between the furan ring and propenamide side chain (present in compounds **24**–**27**) was removed by epoxidation, had a greatly lowered E_7^1 of −335 mV. This analogue, however, showed a slight increase in both aerobic and anaerobic toxicity over **24**–**27**, probably due to the presence of two potential alkylating moieties and showed no hypoxia selectivity. The only 3-nitro analogue synthesized, **31**, showed no differential cytotoxicity towards hypoxic cells and in fact displayed slightly higher oxic toxicity. The E_7^1 was measured for this compound and found to be <−600 mV. Further 3-nitro analogues of this type were therefore not investigated in view of probable similarly low electron affinities.

Despite promising in vitro radiosensitization results with this series of nitrofurans, and some improvement over nitrofurazone(**3**), they were ineffective as radiosensitizers of murine KHT tumors in vivo (Figure 2), and no greater killing of cells could be achieved than was apparent with radiation alone. Indeed, slight radioprotection appeared to occur 5–15 min after administration of **18**, **20**, and **25**, possibly as a consequence of unknown physiological effects of these agents. Only **16** showed a consistent but small radiosensitizing effect. Together with possible poor distribution and tumor uptake, the ineffectiveness of the compounds as radiosensitizers in vivo could be a consequence of rapid metabolism and/or interactions of the compounds with noncritical targets in the animals, as has been postulated previously with some other nitrofurans.[16,17] This could involve, for example, the reaction of metabolized nitrofurans with cellular thiol groups, although in culture medium we have not found these compounds to be reactive towards the thiols glutathione and *N*-acetylcysteine.

In conclusion, these nitrofurans show little promise as mixed-function radiosensitizers of clinical potential unless the high oxic toxicity can be overcome and significant radiosensitizing activity in vivo can be demonstrated. It is pertinent to note, however, that the doses of the nitrofurans which could be administered to mice were relatively low compared to doses used for similar nitroimidazoles, even with those compounds which were not limited by poor solubility. The relatively high toxicity of these compounds toward oxic cells in vitro may lead to dose-limiting toxicity towards normal tissues in vivo. The design of nitrofurans and other nitroheteroarenes with lower (more negative) E_7^1 values may reduce oxic toxicity and could lead to greater hypoxia selectivity. This approach is currently being investigated in this laboratory.

Experimental Section

Chemical Synthesis. NMR Spectra were obtained at 60 MHz with a Jeol PMX60SI spectrometer with $SiMe_4$ as internal standard. Mass spectra were carried out on a Finnigan 4500 instrument in the electron-impact mode. Melting points are corrected. CH_2Cl_2 was purified by passing through basic alumina, drying over $CaCl_2$, and distilling prior to use. Tetrahydrofuran (THF) was distilled from CaH_2; other solvents were unpurified commercial grades unless stated otherwise. Commercially available 3-chloroperbenzoic acid (MCPBA) was further purified by washing with phosphate buffer (pH 7) and drying the residue at reduced pressure in the presence of P_2O_5. Elemental analyses were determined by Elemental Microanalysis Ltd., Okehampton, UK. Solutions in organic solvents were dried by treatment with anhydrous Na_2SO_4 and filtration. Solvents were removed by evaporation under reduced pressure. All compounds were racemic. The one-electron reduction potentials (E_7^1) of selected compounds (Table I) were determined by using pulse-radiolysis techniques as previously described.[18]

Prop-2-enyl 5-Nitrofuran-2-carboxylate (15). 5-Nitro-2-furoyl chloride (0.5 g, 2.85 mmol) in CH_2Cl_2 (3 mL) was added to allyl alcohol (1.4 g, 24 mmol) and Et_3N (3 mL) in CH_2Cl_2 (5 mL) at 0 °C under N_2. The solution was stirred at 0 °C for 1 h, poured onto ice (50 g), and extracted with CH_2Cl_2 (50 mL). The organic layer was washed with 5% aqueous $NaHCO_3$ and H_2O and dried. The solvent was evaporated, and the residue was recrystallized from EtOH to give **15** (0.35 g, 62%) as pale yellow prisms: mp 71–71.5 °C; NMR ($CDCl_3$) δ 4.8 (d, J = 6 Hz, 2 H, allylic CH_2), 5.2–6.2 (m, 3 H, olefinic-H), 7.25 (s, 2 H, furan-H). Anal. ($C_8H_7NO_5$) C, H, N.

Oxiranylmethyl 5-Nitrofuran-2-carboxylate (16). Dried 3-chloroperbenzoic acid (7.3 g, 42 mmol) in CH_2Cl_2 (75 mL) was added to **15** (3.5 g, 17.7 mmol) in CH_2Cl_2 (30 mL). The solution was heated under gentle reflux for 1.5 h before being washed with 10% aqueous Na_2SO_3 (100 mL), 5% aqueous $NaHCO_3$ (twice), H_2O, and saturated aqueous NaCl. The organic layer was then dried, and the solvent was evaporated. The residue was recrystallized from EtOH to give **16** (2.3 g, 61%) as a colorless solid: mp 98–99 °C; NMR ($CDCl_3$) δ 2.7 (dd, J = 4.5 and 3 Hz, 1 H) and 2.9 (t, J = 4.5 Hz, 1 H), oxiranyl 3-CH_2, 3.25 (m, 1 H, oxiranyl 2-H), 4.2 (dd, J = 12 and 6 Hz, 1 H), and 4.7 (dd, J = 12 and 3 Hz, 1 H), CO_2CH_2, 7.25 (s, 2 H, furan-H). Anal. ($C_8H_7NO_6$) C, H, N.

2,2-Dimethyl-1-(5-nitro-2-furoyl)aziridine (17). Epoxide ester **16** (1.0 g, 5 mmol) was stirred at 20 °C with 2,2-dimethylaziridine (4.0 g, 56.3 mmol) for 5 h. Excess aziridine was then evaporated, and the residue was recrystallized from EtOH to give **17** (0.6 g, 57%) as a pale yellow solid: mp 104–106 °C dec; NMR ($CDCl_3$) δ 1.5 (s, 6 H, aziridine-$(CH_3)_2$), 2.45 (s, 2 H, aziridine CH_2), 7.2 (d, J = 3.5 Hz, 1 H, furan-H), 7.3 (d, J = 3.5 Hz, 1 H, furan-H). Anal. ($C_9H_{10}N_2O_4$) C, H, N.

***N*-(1,1-Dimethyl-2-hydroxyethyl)-5-nitrofuran-2-carboxamide (18).** 5-Nitro-2-furoyl chloride (1.5 g, 8.6 mmol) in CH_2Cl_2 (10 mL) was added to 2-amino-2-methylpropan-1-ol (10 mL, ca. 100 mmol) and Et_3N (10 mL) in CH_2Cl_2 (30 mL). The mixture was stirred at 25 °C for 0.5 h and was then washed with H_2O and dried. The solvent was evaporated, and the residue was recrystallized twice from CH_2Cl_2 to afford **18** (1.35 g, 69%) as a colorless solid: mp 110–112 °C; NMR ($CDCl_3$) δ 1.4 (s, 6 H, $(CH_3)_2$), 3.65

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(17) Sutherland, R. M.; Durand, R. E. *Int. J. Radiat. Biol.* **1972**, *22*, 613.
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(s, 2 H, CH_2OH), 6.5 (br, 1 H, NH), 7.2 (d, J = 3.5 Hz, 1 H, furan-H), 7.3 (d, J = 3.5 Hz, 1 H, furan-H). Anal. ($C_9H_{12}N_2O_5$) C, H, N.

5-Nitro-*N*-(prop-2-enyl)furan-2-carboxamide (19). 5-Nitro-2-furoyl chloride (5.0 g, 28.5 mmol) in CH_2Cl_2 (50 mL) was added dropwise to allylamine (12 mL, 276 mmol) and Et_3N (30 mL) in CH_2Cl_2 (50 mL) at 0 °C under N_2, and the solution was stirred at 0 °C for a further 0.75 h. The mixture was washed with 5% aqueous $NaHCO_3$ and saturated aqueous NaCl and was dried. Evaporation of the solvent and recrystallization from EtOH gave **19** (3.3 g, 59%) **5** as pale yellow needles: mp 64–65.5 °C; NMR ($CDCl_3$) δ 4.1 (br t, J = 6 Hz, 2 H, allylic CH_2), 5.2 (br d, J = 13 Hz, 1 H) and 5.3 (br d, J = 17 Hz, 1 H) olefinic CH_2, 5.6–6.2 (m, 1 H, allylic 2-H), 7.0 (br, 1 H, NH), 7.25 (d, J = 3.5 Hz, furan-H), 7.35 (d, J = 3.5 Hz, furan-H). Anal. ($C_8H_8N_2O_4$) C, H, N.

***N*-(Oxiranylmethyl)-5-nitrofuran-2-carboxamide (20).** Epoxidation of **19** was carried out as described for the preparation of **16**. The product was recrystallized from EtOH to afford **20** (1.14 g, 53%) as colorless prisms: mp 110–112 °C; NMR ($CDCl_3$) δ 2.6–4.1 (m, 5 H, oxirane-CH_2 and oxiranyl-H), 6.9 (br, 1 H, NH), 7.2 (d, J = 3.5 Hz, furan-H), 7.3 (d, J = 3.5 Hz, furan-H). Anal. ($C_8H_8N_2O_5$) C, H, N.

***N*-(3-(2,2-Dimethyl-1-aziridinyl)-2-hydroxypropyl)-5-nitrofuran-2-carboxamide (21).** Epoxide **20** (0.2 g, 0.94 mmol) was heated under reflux with 2,2-dimethylaziridine (0.2 g, 2.8 mmol) and Et_3N (0.03 mL) in THF (3 mL) for 5 h. The solvent was then evaporated, and the residue was purified by chromatography (silica gel (75 g); EtOH/Et_3N (99:1, v/v)). Recrystallization from EtOH/Et_3N (99:1) afforded **21** (0.07 g, 26%) as a pale yellow solid: mp 94–95 °C; NMR ($CDCl_3$) δ 1.2–1.3 (m, 7 H, aziridine-$(CH_3)_2$ and aziridine-H), 1.85 (d, J = 3 Hz, 1 H, aziridine-H), 2.3–2.6 (m, 2 H, $CHCH_2$ aziridine), 3.6–3.9 (m, 4 H, $NHCH_2CHOH$), 7.20 (d, J = 3.5 Hz, furan-H), 7.25 (d, J = 3.5 Hz, furan-H), 7.8 (br, 1 H, NH). Anal. ($C_{12}H_{17}N_3O_5$) C, H, N.

***N*-(3-(*cis*-2,3-Dimethyl-1-aziridinyl)-2-hydroxypropyl)-5-nitrofuran-2-carboxamide (22).** Epoxide **20** was treated with *cis*-2,3-dimethylaziridine as for the preparation of **21** above to give **22** (0.32 g, 32%) as a pale yellow solid: mp 87–89 °C; NMR ($CDCl_3$) δ 1.2 (d, J = 6 Hz, 6 H, 2 × aziridine-CH_3), 1.5 (m, 2 H, 2 × aziridine-H), 2.3 (dd, J = 13 and 5 Hz, 1 H), and 2.45 (dd, J = 13 and 5 Hz, 1 H) $CHCH_2$ aziridine, 3.6–4.0 (m, 4 H, $NHCH_2CHOH$), 7.15 (d, J = 3.5 Hz, furan-H), 7.25 (d, J = 3.5 Hz, furan-H), 7.7 (br, 1 H, NH); MS, m/z 284 $(M + H)^+$, 266, 240, 139, 84 (100). Anal. ($C_{12}H_{17}N_3O_5$) C, H, N.

***N*-(3-((2-Bromo-1,1-dimethylethyl)amino)-2-hydroxypropyl)-5-nitrofuran-2-carboxamide Hydrobromide (23).** Aziridine **21** (0.1 g, 0.35 mmol) was treated with 48% aqueous HBr (2 mL) at 25 °C for 10 min, and the excess reagent was evaporated under reduced pressure at 40 °C. H_2O (2 mL) was added and was evaporated; this operation was repeated. A small volume of EtOH was added to the residue, and the product was precipitated with Et_2O and recrystallized from aqueous acetone to afford **23** (0.05 g, 40%) as a yellow solid: mp 115–117 °C; NMR ($CDCl_3$) δ 1.8 (s, 6 H, $(CH_3)_2$), 3.4 (m, 2 H, $CHCH_2^+NH_2$), 3.8–4.5 (m, 4 H, $NHCH_2CHOH$), 4.2 (s, 2 H, CH_2Br), 7.6 (s, 2 H, furan-H). Anal. ($C_{12}H_{19}N_3O_5Br_2 \cdot 0.5H_2O$) C, H, N.

3-(5-Nitrofuran-2-yl)-*N*-(prop-2-enyl)propenamide (24). 5-Nitro-2-furaldehyde (5.4 g, 40 mmol) and dry powdered NaOAc (2.0 g, 40 mmol) were added to acetic anhydride (10 mL), and the mixture heated was at 140–150 °C for 2.5 h. The dark solution was then cooled to 80 °C, and H_2O (100 mL) was added, together with decolorizing charcoal, and the solution was boiled under reflux for 10 min. The hot solution was filtered and acidified with 2 M aqueous HCl. Cooling afforded 3-(5-nitrofuran-2-yl)propenoic acid (4.2 g, 57%) as a pale brown solid: mp 228–230 °C, (lit.[19] mp 235–236 °C). This material (1.83 g, 10 mmol) was boiled under reflux in $SOCl_2$ (3 mL) and dimethylformamide (0.1 mL) for 3.5 h. The $SOCl_2$ was evaporated, and the resulting acyl chloride, in CH_2Cl_2 (20 mL), was added dropwise under N_2 to allylamine (5 mL, ca. 70 mmol) and Et_3N (12 mL) in CH_2Cl_2 (20 mL) at 0 °C. The solution was stirred at 0 °C for 1.5 h before being washed with H_2O, 5% aqueous $NaHCO_3$, and saturated aqueous NaCl and dried. The solvent was removed, and the residue was twice recrystallized from CH_2Cl_2 to give **24** (1.1 g, 50%) as pale yellow needles: mp 168–169 °C; NMR ($CDCl_3$) δ 4.1 (dt, J = 7 and 1 Hz, 2 H, allylic CH_2), 5.20 (br d, J = 13 Hz, 1 H), and 5.25 (br d, J = 18 Hz, 1 H), and 5.6–6.2 (m, 1 H), $CH{=}CH_2$, 6.2 (br t, $J \approx$ 7 Hz, 1 H, NH), 6.7 (d, J = 16 Hz, 1 H, furan-CH=C*H*), 6.7 (d, J = 3.5 Hz, 1 H, furan 3-H), 7.3 (d, J = 3.5 Hz, 1 H, furan 4-H), 7.4 (d, J = 16 Hz, 1 H, furan-C*H*=CH). Anal. ($C_{10}H_{10}N_2O_4$) C, H, N.

***N*-(Oxiranylmethyl)-3-(5-nitrofuran-2-yl)propenamide (25).** Epoxidation of **24** (1.0 g, 4.5 mmol) was carried out as described for compounds **16** and **20** but with an extended reaction time of 2.5 h and purification by column chromatography (silica gel; CH_2Cl_2) to afford, after recrystallization from EtOH, **25** (0.5 g, 47%) as yellow needles: mp 175–177 °C; H NMR ($CDCl_3$) δ 2.65 (dd, J = 5 and 3 Hz, 1 H) and 2.85 (t, J = 5 Hz, 1 H), and 3.2 (m, 1 H) 3 × oxirane-H, 3.3–4.1 (m, 2 H, NCH_2), 6.3 (br, 1 H, NH), 6.65 (d, J = 16 Hz, 1 H, furan-CH=C*H*), 6.7 (d, J = 3.5 Hz, 1 H, furan 3-H), 7.3 (d, J = 3.5 Hz, 1 H, furan 4-H), 7.4 (d, J = 16 Hz, 1 H, furan-C*H*=CH); MS, m/z 239 $(M + H)^+$, 192, 166 (100), 134. Anal. ($C_{10}H_{10}N_2O_5$) C, H, N.

***N*-(3-(2,2-Dimethyl-1-aziridinyl)-2-hydroxypropyl)-3-(5-nitrofuran-2-yl)propenamide (26).** **25** (0.2 g, 0.84 mmol) was heated with 2,2-dimethylaziridine (0.2 g, 2.8 mmol) and Et_3N (0.03 mL) in THF (3 mL) under reflux for 6 h. The solvent was then removed, and the residue was purified by centrifugally accelerated layer chromatography (silica gel; EtOH/Et_3N (99:1 v/v)) to give **26** (0.15 g, 58%) as a pale yellow solid: mp 149–150 °C; NMR ($CDCl_3$) δ 1.3–1.5 (m, 7 H, $C(CH_3)_2$ and aziridine-H), 1.85 (d, J = 3 Hz, 1 H, aziridine-H), 2.3–2.6 (m, 2 H, $CHCH_2$-aziridine), 3.8–4.2 (m, 4 H, $NHCH_2CHOH$), 6.65 (d, J = 16 Hz, 1 H, furan-CH=C*H*), 6.7 (d, J = 3.5 Hz, 1 H, furan 3-H), 7.3 (d, J = 3.5 Hz, 1 H, furan 4-H), 7.4 (d, J = 16 Hz, 1 H, furan-C*H*=CH); MS m/z 310 $(M + H)^+$ (100), 85, 70, 56. Anal. ($C_{14}H_{19}N_3O_5 \cdot 0.33H_2O$) C, H, N.

***N*-(3-(*cis*-2,3-Dimethyl-1-aziridinyl)-2-hydroxypropyl)-3-(5-nitrofuran-2-yl)propenamide (27).** Epoxide **25** was treated with *cis*-2,3-dimethylaziridine as for the preparatin of **26** above to afford, after recrystallization from EtOH, **27** (0.15 g, 48%) as a yellow solid: mp 145–146 °C; NMR ($CDCl_3$) δ 1.2 (d, J = 6 Hz, 6 H, 2 × aziridine-CH_3), 1.6 (m, 2 H, 2 × aziridine-H), 2.5 (m, 2 H, $CHCH_2$-aziridine), 3.1–3.8 (m, 4 H, $NHCH_2CHOH$), 6.65 (d, J = 16 Hz, 1 H, furan-CH=C*H*), 6.7 (d, J = 3.5 Hz, 1 H, furan 3-H), 7.3 (d, J = 3.5 Hz, 1 H, furan 4-H), 7.4 (d, J = 16 Hz, 1 H, furan-C*H*=CH) 7.5 (br, 1 H, NH). Anal. ($C_{14}H_{19}N_3O_5 \cdot 0.33H_2O$) C, H, N.

***N*-(3-(*cis*-2,3-Dimethyl-1-aziridinyl)-2-hydroxypropyl)-2,3-epoxy-3-(5-nitrofuran-2-yl)propenamide (28).** A crude sample of **25** (0.5 g, ca. 2.4 mmol) and 3-chloroperbenzoic acid (0.5 g, 2.9 mmol) was boiled under reflux with Et_3N (0.24 mL) and *cis*-2,3-dimethylaziridine (0.4 mL, ca. 8 mmol) in THF (6 mL) for 6 h. The solvent and excess aziridine were then evaporated. The residue was subjected to column chromatography (silica gel; EtOAc/Et_3N (99:1, v/v)) and recrystallization from EtOH to give **28** (0.25 g, 34%) as a yellow solid: mp 74–75 °C; NMR ($CDCl_3$) δ 1.2 (d, J = 6 Hz, 6 H, 2 × aziridine-CH_3), 1.6 (m, 2 H, 2 × aziridine-H), 2.5–2.6 (m, 2 H, $CHCH_2$-aziridine), 2.7–4.0 (m, 6 H, $NHCH_2CHOH$ and 2 × oxirane-H), 6.5 (d, J = 3.5 Hz, 1 H, furan 3-H), 7.05 (br, 1 H, NH), 7.25 (d, J = 3.5 Hz, 1 H, furan 4-H). Anal. $C_{14}H_{19}N_3O_6$ C, H, N.

2-Methyl-5-nitro-*N*-(prop-2-enyl)furan-3-carboxamide (29). 2-Methyl-5-nitrofuran-3-carboxylic acid (prepared according to the methods of Gilman et al.[13]) (2.0 g, 11.7 mmol) was stirred for 1.5 h at 0 °C with ethyl chloroformate (1.26 g, 11.7 mmol) and Et_3N (1 mL) in CH_2Cl_2 (20 mL). Allylamine (2.0 g, 35.1 mmol) was then added and stirring continued for 1 h at 0 °C. The solution was then poured onto ice (50 g), stirred for 0.5 h, and extracted with Et_2O (2 × 50 mL). The combined organic extracts were washed with 10% aqueous $NaHCO_3$ (twice) and H_2O and dried, and the solvent was removed. The residue was twice recrystallized from EtOH to give **29** (1.5 g, 61%) as colorless needles: mp 100–101 °C; NMR ($CDCl_3$) δ 2.8 (s, 3 H, furan-CH_3), 4.1 (br t, J = 6.5 Hz, 2 H, allylic-CH_2), 5.05–6.3 (m, 3 H, $CH{=}CH_2$), 6.35 (br, 1 H, NH), 7.45 (s, 1 H, furan 4-H). Anal. ($C_9H_{10}N_2O_4$) C, H, N.

***N*-(Oxiranylmethyl)-2-methyl-5-nitrofuran-3-carboxamide (30).** Oxirane **30** was prepared as described for compounds

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Table II. In Vivo Studies. Maximum Doses Administered to Mice

compound	vol injected per 25 g of body wt, mL	max dose, mmol kg^{-1} [c]	limiting factor
16	1.0	0.12 (25 mg kg^{-1})	solubility[a]
18	0.5	0.44 (100 mg kg^{-1})	toxicity[b]
20	0.5	0.24 (50 mg kg^{-1})	toxicity[b]
25	1.0	0.11 (25 mg kg^{-1})	solubility[a]

[a] Injected as suspensions. [b] No acute, severe, or persistent physical or behavorial effects were apparent in C3H/He mice with any of the doses shown in the table. However, following injection of 18, piloerection, an increased respiration rate, and decreased locomotor activity were observed in the mice for approximately 1 h after injection. After higher doses of any of the drugs limited by toxicity severe tremors and convulsions occurred, from which the mice did not recover. [c] Injected ip.

16 and 20 from 0.5 g (2.5 mmol) of 29. Recrystallization from EtOH gave 30 (0.3 g, 53%) as colorless needles: mp 129–130 °C; NMR ($CDCl_3$) δ 2.8 (s, 3 H, furan-CH_3), 2.75–4.1 (m, 5 H, NCH_2 and 3 × oxirane-H), 6.5 (br, 1 H, NH), 7.45 (s, 1 H, furan 4-H). Anal. ($C_9H_{10}N_2O_5$) C, H, N.

2-Methyl-3-nitro-*N*-(prop-2-enyl)furan-5-carboxamide (31). 2-Methyl-3-nitrofuran-5-carboxylate (prepared by the method of Rinkes[14]) (0.25 g, 1.4 mmol) was stirred with allylamine (3 mL, 69 mmol) and dicyclohexylcarbodiimide (2.1 g, 10 mmol) in THF (7 mL) at 25 °C for 12 h. Excess amine was then evaporated after filtration and the residue was purified by chromatography (silica gel; EtOAc/$CHCl_3$ (1:1, v/v)) to give 31 (0.1 g, 35%) as pale yellow prisms: mp 55–56 °C; NMR ($(CD_3)_2SO$) δ 2.6 (s, 3 H, furan-CH_3), 4.0 (m, 2 H, allylic CH_2), 5.2–5.8 (m, 3 H, $CH{=}CH_2$), 7.6 (s, 1 H, furan 4-H), 8.5 (br t, J = 7 Hz, 1 H, NH). Anal. ($C_9H_{10}N_2O_4$) C, H, N.

Biological Methods. The radiosensitization studies in vitro were carried out as described previously with use of Chinese hamster V79-379A cells.[8] The methods for determining selective toxicity to hypoxic V79-379A cells using the MTT assay are also described elsewhere.[20]

On the basis of results from experiments in vitro, compounds 16, 18, 20, and 25 were selected for evaluation in vivo in C3H/He mice. The compounds were injected as suspensions or solutions in phosphate-buffered saline (pH 7.3). TLC analyses were carried out to show that the compounds were unchanged at the time of injection. Initial studies were carried out to determine the maximum doses of compounds which could be administered (Table II). Subsequently, the maximum single doses of each compound which could be administered (according to toxicity or solubility) were injected at various times (5–90 min) before local irradiation of subcutaneous KHT sarcomas with a 10-Gy dose of X-rays. Tumors were excised 24 h later and clonogenic assays performed in vitro to determine the survival of tumor cells.[8]

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PUBLICATION 39

Synthesis and Evaluation of 1-[3-(2-Haloethylamino)propyl]-2-nitroimidazoles as Pro-Drugs of RSU 1069 and its Analogues which are Radiosensitizers and Bioreductively-Activated Cytotoxins

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Synthesis and Evaluation of α-[[(2-Haloethyl)amino]methyl]-2-nitro-1*H*-imidazole-1-ethanols as Prodrugs of α-[(1-Aziridinyl)methyl]-2-nitro-1*H*-imidazole-1-ethanol (RSU-1069) and Its Analogues Which Are Radiosensitizers and Bioreductively Activated Cytotoxins

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α-[(1-Aziridinyl)methyl]-2-nitro-1*H*-imidazole-1-ethanols, of general formula $ImCH_2CH(OH)CH_2\overline{NCR^1R^2CR^3R^4}$, where Im = 2-nitroimidazole and R^1, R^2, R^3, R^4 = H, Me, are radiosensitizers and selective bioreductively activated cytotoxins toward hypoxic tumor cells in vitro and in vivo. Treatment of the aziridines with hydrogen halide in acetone or aqueous acetone gave the corresponding 2-haloethylamines of general formula $ImCH_2CH(OH)CH_2^+NH_2CR^1R^2CR^3R^4X\ X^-$, where R^1, R^2, R^3, R^4 = H, Me, and X = F, Cl, Br, I. These 2-haloethylamines were evaluated as prodrugs of the parent aziridines. The rates of ring closure in aqueous solution at pH ~6 were found to increase with increasing methyl substitution and to depend on the nature of the leaving group (I ~ Br > Cl ≫ F). A competing reaction of $ImCH_2CH(OH)CH_2^+NH_2CH_2CH_2X\ X^-$ (X = Cl, Br) with aqueous HCO_3^- ions gives 3-[2-hyroxy-3-(2-nitro-1*H*-imidazol-1-yl)propyl]-2-oxazolidinone. The activities of these prodrugs as radiosensitizers or as bioreductively activated cytotoxins were consistent with the proportion converted to the parent aziridine during the course of the experiment. α-[[(2-Bromoethyl)amino]methyl]-2-nitro-1*H*-imidazole-1-ethanol (RB 6145, 10), the prodrug of α-[(1-aziridinyl)methyl]-2-nitro-1*H*-imidazole-1-ethanol (RSU-1069, 3), is identified as the most useful compound in terms of biological activity and rate of ring closure under physiological conditions.

Introduction

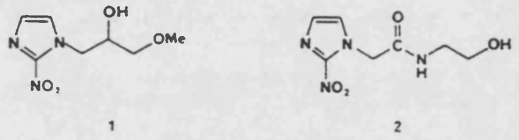
It is generally known that many solid tumors contain areas of diminished oxygen supply. While the underlying reasons for this hypoxia can vary depending upon the type and environment of the tumor, this phenomenon provides a potential basis for selectivity of cancer chemotherapeutic agents towards tumors. Compounds which are anaerobically reduced to a cytotoxin should be more cytotoxic to hypoxic tumor cells than to oxygenated normal tissues.[1] Many nitroheterocyclic compounds, such as the 2-nitroimidazoles, owe their selective cytotoxicity toward hypoxic cells within tumors to their bioreductive properties.[2] They are activated in vivo by anaerobic, enzymatic reduction to form metabolites which are considerably more cytotoxic than the parent compound from which they were derived.

Nitroimidazoles also act as hypoxic cell radiosensitizers, acting primarily by fast free-radical mechanisms.[3] The agents increase the sensitivity to radiation of the normally radiation-resistant hypoxic cells but have little or no effect on the response of well-oxygenated cells to radiation. The ability of these compounds to act as radiosensitizers[4] and hypoxic cell cytotoxins is related to their reduction potentials, although the mechanisms of the two effects are different.

Misonidazole (1)[5] has undergone extensive clinical evaluation as a radiosensitizer, but cumulative neurotoxicity severely limits the dosage which may be administered. An equally potent but less toxic analogue, etanidazole (2),[6] is in phase III trials. An alternative approach has been to develop compounds with greater potency than 1 or 2 (Chart I). An example is the dual functional agent α-[(1-aziridinyl)methyl]-2-nitro-1*H*-imidazole-1-ethanol (3), which combines the 2-nitroimidazole ring with an alkylating moiety, resulting in a powerful hypoxic cell radiosensitizer and bioreductively activated cytotoxic agent both in vitro and in vivo.[7-9] This aziridine 3 was selected from a series of compounds based upon its partition coefficient and activity in model tumors. The ability of 3 to sensitize hypoxic tumor cells to the lethal effects of radiation at 10–12 times less than the dose of 1 or 2 in experimental animal models led to its investigation clinically. Unfortunately, in early phase I trials, gastric toxicity (emesis) was observed.[10] The maximum tolerated dose achievable in humans was considered to be too low to achieve a usefully high degree of radiosensitization. This led to a search for equally active but less toxic analogues.

This study describes the synthesis, stability, and development of a series of [(haloethyl)amino]propyl-2-nitroimidazoles derived from 3 and its analogues. Preliminary biological studies of these compounds as radios-

Chart I. Structures of the Hypoxic Cell Radiosensitizers Misonidazole (1) and Etanidazole (2)



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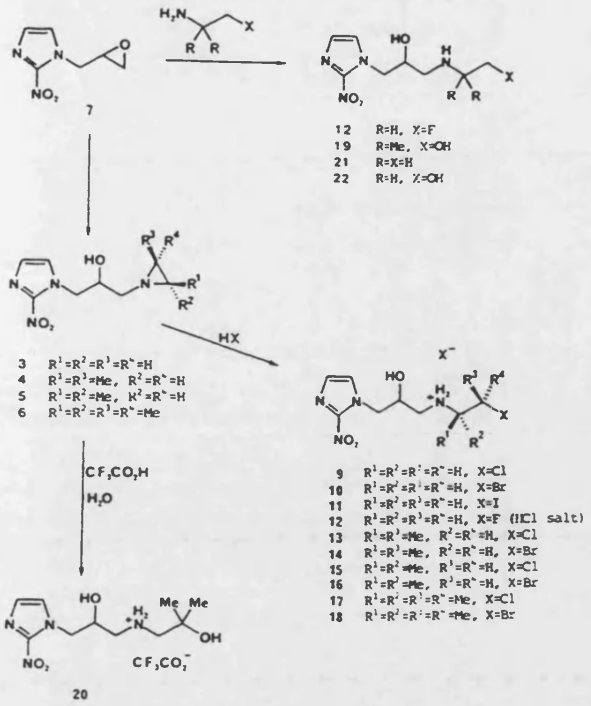
Scheme I. Synthesis of 2-Haloethylamines **9–18**, 2-Hydroxyethylamines **19**, **20**, and **22**, and Ethylamine **21**



ensitizers are also provided.

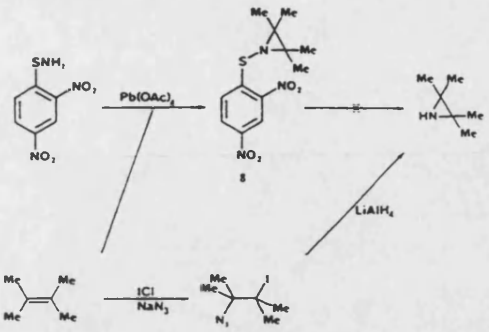
Chemistry

Compounds **3–6** were prepared as described previously by us[8,11,12] from oxirane **7** and the corresponding aziridine (Scheme I). The overall yield of **4** was increased considerably with stereochemically pure *cis*-2,3-dimethylaziridine prepared from *cis*-2,3-epoxybutane by the method of Dickey et al.[13] Alternative procedures for the synthesis of 2,2,3,3-tetramethylaziridine, used in the preparation of **6**, were explored, in order to avoid the use of the expensive, noxious, and unreliable nitrosyl chloride.[14,15]

The cyclization of 2-amino alcohols via the *O*-sulfates fails for the more substituted examples. The insertion of [(2,4-[dinitrophenyl)sulfenyl]nitrene, generated from 2,4-dinitro benzenesulfonamide and lead tetraacetate,[16] into the double bond of 2,3-dimethylbut-2-ene gave 1-[2,4-dinitrophenyl)thio]-2,2,3,3-tetramethylaziridine (**8**) in high yield (Scheme II). However, reductive cleavage of the S–N bond could not be achieved in preparatively useful procedures. Addition of iodine azide, formed in situ from sodium azide and iodine monochloride, across the double bond of 2,3-dimethylbut-2-ene, reduction of the resultant azide, and concomitant ring closure furnished the aziridine in acceptable yield[17] (Scheme I).

The 2-haloethylamines **9–18** were obtained from the corresponding aziridines **3–6** by acid-catalyzed nucleophilic ring opening with the appropriate hydrogen halide, as shown in Scheme II. The yields were generally greater if anhydrous conditions were employed, thus avoiding the possibility of nucleophilic attack by water leading to the corresponding 2-hydroxyethylamines.

Scheme II. Synthetic Approaches to 2,2,3,3-Tetramethylaziridine



Compounds **13** and **14** have the threo relative stereochemistry in the haloalkylamine moiety but were isolated as mixtures of diastereoisomers with respect to the chiral center adjacent to the hydroxy group. Proton NMR at 300 MHz and HPLC analysis revealed that **13** consisted of such diastereoisomers in the approximate 1:1 ratio, indicating that the electrophilic centers in protonated **4**, which are prochiral, are roughly equivalent in reactivity and/or steric crowding in the reacting conformation. HPLC analysis of **14** also indicated the presence of both the analogous diastereomers in similar quantities. Ring-closure of **13** and **14** in the presence of aqueous base gave exclusively *cis*-2,3-dimethylaziridine **4**, showing that both ring-opening and ring-closing reactions proceed with complete inversion at the reacting center.

The correct regioisomeric structures of **15** and **16** as 1,1-dimethyl-2-haloethylamines which are products of attack at CH_2, rather than isomeric 2-halo-2-methylpropylamines resulting from alternative attack at CMe_2, were also confirmed by two pieces of evidence from ^1H NMR spectra. Firstly, the CMe_2CH_2Cl protons of **15**, in solution in 10% deuterotrifluoroacetic acid in deuterium oxide, resonate as a sharp singlet at δ 3.90. In comparison, the spectrum of **19**, in the same solvent, shows a corresponding singlet at δ 3.80 for the CMe_2CH_2OD protons whereas that of isomeric alcohol **20**, prepared in situ from **3** by hydrolytic ring opening in the NMR solvent, contains a singlet for the $^+ND_2CH_2CMe_2OD$ protons at δ 3.40. Secondly, when the spectra were recorded of solutions of these materials in anhydrous protiotrifluoroacetic acid, coupling was evident between the $^+NH_2$ protons and the adjacent CH_2CMe_2 in **20** but was absent from the $^+NH_2CMe_2CH_2OH$ signal of **19**. The corresponding signal of **15** also showed no coupling to the $^+NH_2$ protons. These observed regioselectivities of acid-catalyzed ring opening of 2,2-dimethylaziridines with the weak nucleophilic water and the strong nucleophile chloride are consistent with the reported[18] corresponding reactions of simple *N*-unsubstituted aziridines. The attack of chloride proceeds via a S_N2-type mechanism involving the less hindered CH_2 center; whereas water can only trap the more stable tertiary carbonium ion in a S_N1-like process.

The diastereotropic nature of the $NC(CH_3)_2$ and $ClC(CH_3)_2$ groups of protons of **17** was revealed by proton NMR at 250 MHz in solution in $(CD_3)_2SO$. The $\Delta\delta$ for the pairs of methyl groups were 0.02 and 0.01 ppm, respec-

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Scheme III. "One-Pot" Conversion of 2-Hydroxyethylamines **22** and **19** to 2-Bromoethylamines **10** and **16**

22 ($R^1 = R^2 = H$)[a] **10** ($R^1 = R^2 = H$)[a]

19 ($R^1 = R^2 = Me$)[b] **16** ($R^1 = R^2 = Me$)[b]

[a]Reagents: Me_2SBr_2 [b]Reagents: NBS/Ph_3P

tively, indicating their remoteness from the *CHOH chiral center. Resolution of the corresponding NCH_2CH_2Cl prochiral methylene protons was not possible. However, in the 250- and 300-MHz spectra of **12**, **13**, and **17**, the $^+NH_2$ protons were found to be significantly inequivalent, with $\Delta\delta$ ranging from 0.14 to 0.13 ppm, possibly indicating conformations involving one, but not the other, NH proton in an intramolecular hydrogen bond.

Direct reaction of oxirane **7** with 2-fluoroethylamine or with ethylamine in ethanol provided compounds **12** and **21**, respectively, as shown in Scheme I. However, this method cannot be used to prepare the more reactive haloethylamino compounds **9**–**11** and **13**–**18**, since unavoidable cyclization and/or polymerization of the starting (substituted) 2-haloethylamine occurs.

Attempts to replace directly the primary hydroxyl groups of **19** and **22** by bromine, thus avoiding the relatively toxic and unstable aziridines **3** and **5**, met with some limited success (Scheme III). Treatment of diol **22** with bromodimethylsulfonium bromide by the general procedure of Furukawa et al.,[19] followed by chromatographic isolation and addition of hydrogen bromide, gave bromo compound **10** in 18% yield. The corresponding reaction of diol **19** with triphenylphosphine and *N*-bromosuccinimide according to the method of Ponpipom and Hanessian[20] similarly afforded **16** in 27% yield. However, in view of the extremely rapid cyclization of the free bases of **10** and **16** noted below, it is possible that the materials eluted from the chromatography columns were not these free bases. These "brominating agents" may have effected cyclization of the hydroxyethylamines to the corresponding aziridines **3** and **5** and treatment of these chromatographically purified intermediates with hydrogen bromide may have caused ring-opening as above. Triphenylphosphine dibromide[21] and triphenylphosphine/carbon tetrachloride[22] are known to effect such cyclizations.

Stability of Compounds and Biological Studies "in Vitro"

The first half-lives for the reaction of a series of 2-haloethylamino compounds in phosphate-buffered saline solution (PBS) were determined from the dependence of their rate of loss with time and are shown in Table I for the conditions indicated. With increasing methyl-substitution of the analogues, it was necessary to investigate their degradation at a lower temperature, e.g. 4 °C. Even at this temperature, the tetramethyl analogues **17** and **18** are consumed within the time required for preparation of solutions. The compounds become more stable when the solution pH $\ll$ pK_a of the amino group, the pK_a of which is estimated to be ~7.2 for compounds **9**–**11**. The major product formed in PBS is the corresponding aziridine, the product of ring-closure, as shown in Table I. For compound **10**, it was confirmed that the rate of release of bromide is the same as that for the loss of **10**, indicating that the rate-determining step involves ring closure. These findings are consistent with the degradation occurring predominantly via the free base. From the half-lives given in Table I, it is apparent that the rate of ring closure is influenced by (i) the leaving-group ability of the halide and (ii) the pattern of methyl substitution. The aqueous stability of these agents is in agreement with previous observations with simple substituted haloethylamino compounds.[23]

Table I. Half-Life of the Agents in PBS Aqueous Solution at pH 5.5–6.0 Together with the Major Product Formed at 23 °C

substance	half-life/min	major product	% methanol for HPLC
9	$\gg$300 (~280)[a]	**3**	10
10	30	**3**	10
11	80	**3**	10
13	120	**4**	13.75
14	30[b]	**4**	10
15	76[b]	**5**	25
16	<5[b]	**5**	25
17	$\ll$2[b]	**6**	10
18	$\ll$2[b]	**6**	10

[a]At pH 7.0. [b]At 4 °C.

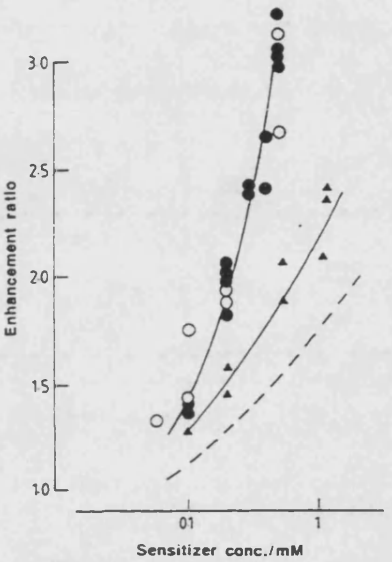

Figure 1. The dependence of hypoxic radiosensitization of V79 cells in vitro on the concentration of **3** (●), **9** (▲), and **10** (○). The dashed line shows the dependence of **1** for comparison.

From assessment of the dependence of hypoxic radiosensitization in vitro for **9** and **10** on concentration, as shown in Figure 1, it is apparent that the active agent in the biological response to **10** is ring-closed aziridine **3**. In contrast, the radiosensitizing efficiency of **9** is less than that of **10** and similar to that of **1**, a conventional 2-nitroimidazole radiosensitizer whose redox potential (E_7^1 = −389 mV)[24] is similar to that of **3**. These findings are consistent with the observation that, at pH 7, **10** but not **9** is cyclized to the aziridine **3** in PBS within a time considerably less than the preirradiation contact period used

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Table II. Concentration of Agent Required To Reduce Cell Survival to 1% following Incubation (3 h) with V79 Mammalian Cells under Aerobic (C_{air}) or Hypoxic (C_{N_2}) Conditions

substance	C_{air}/mM	C_{N_2}/mM	C_{air}/C_{N_2}
9	5	0.35	14.3
10	2.3	0.09	25.5
11	2.4	0.08	30.0
12	35	2.5	14.0
3	0.3	0.003	100.0
22	70	3.5	20.0

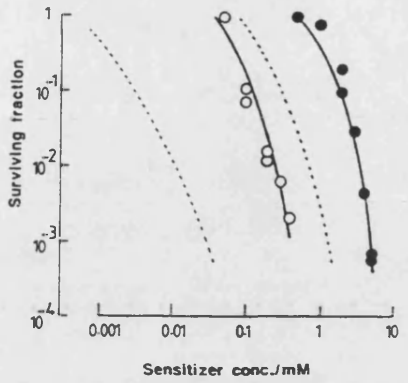


Figure 2. The in vitro cytotoxicity of **10** following exposure (3 h) under hypoxic (○) and aerobic (●) conditions. The dashed line represents the cytotoxicity exhibited by **3** under both gassing conditions.

in the radiosensitization studies in vitro. Further, the similarity of radiosensitizing efficiency of **9** and **1** is consistent with the determined stability of **9** under these conditions. The abilities of the other agents listed in Table I to act in vitro as radiosensitizers was not determined, owing to their rapid conversion in solution at pH 7 to the corresponding aziridine.

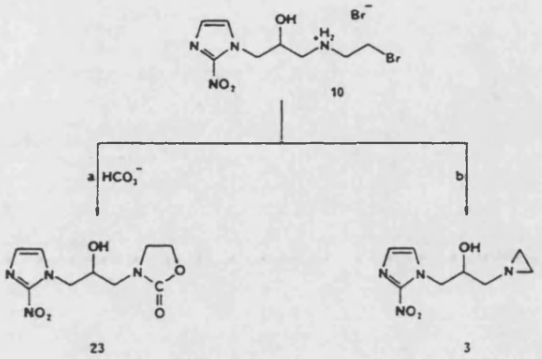
The cytotoxicity of these agents was determined in vitro in V79 cells held in cell culture growth medium at pH 7.4 instead of the PBS used for the radiosensitization studies. In contrast to the similarity of the radiosensitizing efficiency of **10** with that of the corresponding aziridine **3**, it is apparent that the haloethylamines tested (**9–12**) are far less cytotoxic, under both aerobic and hypoxic conditions, than the corresponding aziridine **3** as shown in Table II and in Figure 2. The cytotoxicity of the hydroxyethylamine **22** is shown in Table II for comparison. The other haloethylamines were not tested since their stabilities in solution at pH 7 will result in their conversion within a very short period compared with the contact period of 3 h. From these differences in biological response of **9–12** compared with that of **3**, it is inferred that the reactive haloethylamines undergo additional chemical transformations in cell-culture medium to yield not only **3** but also a compound which is less cytotoxic than **3**.

It was established, from testing several of the individual components of the medium, that **9–11** interact with hydrogen carbonate ion. With a concentration of hydrogen carbonate (24 mM) in PBS equivalent to that in cell-culture medium, it was found that an additional product is formed in competition with formation of the aziridine in the ratios shown in Table III. Increasing the concentration of hydrogen carbonate (24–107 mM) increases the importance of the pathway leading to the product as shown in Scheme IV. This product was identified, by coelution with authentic compound,[25] to be the corresponding oxazolidin-2-one **23**. Formation of oxazolidin-2-ones has previously been shown to occur upon interaction of hydrogen carbonate with simple 2-haloethylamines.[26] Scheme IV shows the various pathways with **10** as an example. For the methylated analogues **13–18**, the rates of cyclization to aziridines were so great as to preclude the formation of detectable quantities of the corresponding oxazolidinones. Thus, whereas methyl substitution enhances substantially the rate of formation of the 3-membered ring owing to the relief of steric crowding in the approach to the transition state, such steric relief is not available in the transition state for formation of the 5-membered cyclic analogue.

Table III. Ratio of Oxazolidinone to Aziridine Formed upon Treatment of the Agents with PBS Aqueous Solution Containing $NaHCO_3$ at pH 7.0–7.5 and 23 °C

substance	[oxazolidinone]/[aziridine]
9	>9.5:1
10	2.3:1
13	<0.05:1

Scheme IV. Competing Reactions of Prodrug **10** in Phosphate-Buffered Saline Solution Containing Hydrogen Carbonate Ions



Since the cytotoxicity experiments are performed at 37 °C, the effect of temperature (20–37 °C) upon the degradation of **10** in PBS containing hydrogen carbonate (24 mM) was determined. The formation of **23** is favored with increasing temperature (step a, Scheme IV). Assuming that **23** shows minimal cytotoxicity over the concentration range of **10** employed, the observed cytotoxicity of **10** in Table II is in reasonable agreement with that resulting from the yield of **3** formed under these biological conditions. Further, the formation of **23** may reduce the cytotoxicity of **3** under hypoxic conditions as has previously been shown for **3** coincubated with **1**.[27]

Biological Assessment of the Compounds in Mice

The maximum tolerated doses (MTD) determined for the compounds administered intraperitoneally (ip) to C3H/He mice are presented in Table IV. The dose-limiting factor for the agents is their toxicities (Table IV) with the exception of **14**, **16**, and **18**, which are limited by their solubilities. Since compounds **13–18** will be converted to the aziridines (see above) within the period required for preparation of the solution and prior to administration, the MTD values for those compounds limited by toxicity are consistent with those determined for the corresponding

(25) Suto, M. U.S. Patent 07/274,209, Nov. 25, 1988.

(26) Rauen, H. M.; Norpoth, K.; Kramer, K.-P. *Arzneim. Forschung* **1965**, *15*, 1048.

(27) Stratford, I. J.; Walling, J. M.; Silver, A. R. J. *Br. J. Cancer* **1986**, *53*, 339.

Table IV. MTD Determined for a Series of Haloethylamine Analogues in C3H Mice

substance	MTD/mmol kg^{-1}	limit to MTD
3	0.4	toxicity
4	3.0	toxicity
6	1.5[a]	toxicity
9	1.5	toxicity
10	1.0	toxicity
13	3.0	toxicity
14	1.0	solubility
15	0.8	toxicity
16	1.0	solubility
17	1.3	toxicity
18	0.7	solubility
21	3.9	toxicity

[a] Administered as a suspension in arachis oil.

aziridines 4–6 (Table IV). In contrast, the MTD values of 9 and 10, which were administered as the haloethylamines, as verified with HPLC, are ~2.5 and 4 times greater than that of the corresponding aziridine 3 but less than the corresponding ethylamino compound 21. The latter compound is stable to ring closure but has a pK_a value which is about 2 pK units greater than that of 9, a property that has previously been shown to modify uptake of drugs of this type.[28] Such comparatively high values of MTD of compounds 9 and 10 are compatible with their partial conversion to a less toxic product as well as to 3 in vivo.

From the chemical studies, it is evident that only 9 and 10, of the haloethylamines reported in Table IV, were sufficiently stable in aqueous solution for direct assessment of their ability to act as radiosensitizers of the KHT sarcoma. The properties in vivo of the other compounds are consistent with their conversion to the corresponding aziridine prior to administration.[29] The dependences of the surviving fraction of clonogenic tumor cells upon ip dose of 9 or 10 are shown in Figure 3 for administration 45 min prior to irradiation of the tumor with X-rays (10 Gy). This dose of radiation kills most of the oxic tumor cells and hence the responses reflect predominantly the modification of the survival of residual clonogenic, hypoxic cells. It is apparent that 10 is slightly less effective than 3 as a radiosensitizer but is about 1 order of magnitude more effective than 9 for an equal dose. From other studies, the effectiveness of 9 to act as a radiosensitizer has been shown to be similar to that of 21 for equivalent administered doses.[29] For compounds 3 and 10, the cell survival falls to a constant value at the highest administered doses used. This value is equivalent to that corresponding to the maximum radiosensitizing effect of oxygen.[30] At the highest tolerated dose of 9, the reduction of cell survival is about 1 order of magnitude less than that observed at the MTD of 3 and 10.

The differences in the effectiveness of 9 and 10 as radiosensitizers could be explained if 23, formed to a greater extent from 9 than from 10 in the presence of hydrogen carbonate (Table III), is a relatively ineffective radiosensitizer in vivo at these concentrations. The ability of 23 to act as a radiosensitizer of hypoxic tumor cells was investigated. Compound 23 was produced from 9 in PBS containing hydrogen carbonate; greater than 95% conversion to 23 was verified by HPLC prior to its administration. As shown in Figure 3, 23 produces little or no radiosensitization at the concentration used and is far less effective than 10 (or 3).

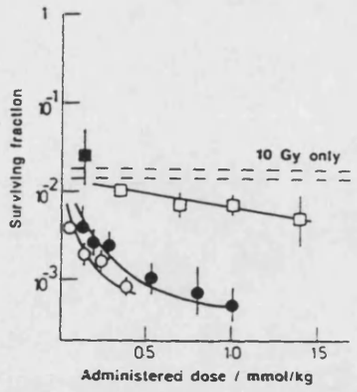


Figure 3. Comparison of the radiosensitization of the KHT sarcoma by various concentrations of 3 (○), 9 (□), 10 (●) and 23 (■) administered ip 45 min before a 10 Gy dose of X-rays.

Conclusions

Treatment of aziridines 3–6 with hydrogen halides under mild conditions provides an efficient route to 2-haloethylamine salts 9–18. With the exception of fluoro compound 12, the corresponding free bases revert to the parent aziridine at rates depending on the nature of the leaving halide and on substitution in the 2-haloethyl moiety. Compounds 9–11 and 13–18 can therefore be considered as prodrugs of 3–6. As the conversions of 13–18 to the parent aziridines are extremely rapid in neutral or slightly acidic aqueous media, the soluble haloethylamine salts may be useful in the formulation of solutions of 4–6 for clinical use. However, the corresponding conversions of 9 and 10 to 3 are sufficiently slow at pH 5 to enable direct assessment of their biological activities in vivo. Studies with the murine KHT tumor demonstrate that the efficiency of 9 to act as a radiosensitizer is similar to that of 21, reflecting its relative resistance to ring closure under the experimental conditions. In contrast, 10, which converted in significant yield to 3 under the experimental conditions, shows an efficiency of radiosensitization comparable to that of 3 but is less toxic. Of the compounds tested for biological activity, 10, acting as a prodrug of 3, may show considerable promise as a radiosensitizer with an increased therapeutic ratio. The ability of this series of compounds to act as bioreductively activated cytotoxins in vivo is reported elsewhere.[29]

Experimental Section

Synthetic Chemistry. Unless otherwise stated, NMR spectra were obtained at 60 MHz with a JEOL PMX60SI spectrometer using $SiMe_4$ as internal standard. Other NMR spectra were obtained at 300 MHz (Bruker AC300) and at 250 MHz (Bruker AM250). A Philips PU9516 instrument furnished the IR spectra. Melting points are uncorrected. Elemental microanalyses were carried out by Butterworths Laboratories Ltd., Middlesex, UK, and Elemental Microanalysis Ltd., Okehampton, UK. *N,N*-Dimethylformamide (DMF) was distilled under reduced pressure from molecular sieves before use. Ether refers to diethyl ether. Solvents were evaporated under reduced pressure. α-[[(2-Hydroxyethyl)amino]methyl]-2-nitro-1*H*-imidazole-1-ethanol (22) was prepared as described previously.[31]

(28) Adams, G. E.; Ahmed, I.; Clarke, E. D.; O'Neill, P.; Parrick, J.; Stratford, I. J.; Wallace, R. G.; Wardman, P.; Watts, M. E. *Int. J. Radiat. Biol.* **1980**, *38*, 613.
(29) Cole, S.; Stratford, I. J.; Adams, G. E.; Fielden, E. M.; Jenkins, T. C. *Radiat. Res.* **1990**, in press.
(30) Stratford, I. J.; Adams, G. E.; Godden, J.; Howells, N. *Int. J. Radiat. Biol.* **1989**, *55*, 411.
(31) Silver, A. R. J.; McNeil, S. S.; O'Neill, P.; Jenkins, T. C.; Ahmed, I. *Biochem. Pharmacol.* **1986**, *35*, 3923.

α-[(*cis*-2,3-Dimethyl-1-aziridinyl)methyl]-2-nitro-1*H*-imidazole-1-ethanol (4). Oxirane **7** was treated with *cis*-2,3-dimethyl aziridine[13] in boiling ethanol by the general method of Adams et al.[8,11,12] to afford **4** (54%) as an off-white solid: mp 84–85 °C (lit.[12] mp 84–85 °C); NMR (300 MHz; $CDCl_3$) δ 1.10 (d, J = 5.5 Hz, 3 H, CH_3), 1.11 (d, J = 5.5 Hz, 3 H, CH_3), 1.53 (dq, J = 6.6 Hz, J = 5.5 Hz, 1 H, aziridine-H), 1.58 (dq, J = 6.6 Hz, J = 5.5 Hz, 1 H, aziridine-H), 1.8 (br, 1 H, OH), 2.32 (dd, J = 12.0 Hz, J = 4.1 Hz, 1 H), and 2.49 (dd, J = 12.0 Hz, J = 7.7 Hz, 1 H) (aziridine-CH_2), 4.06 (ddt, J = 4.1 Hz, J = 2.7 Hz, J ≈ 8 Hz, 1 H, C*H*OH), 4.29 (dd, J = 13.8 Hz, J = 8.0 Hz, 1 H) and 4.67 (dd, J = 13.8 Hz, J = 2.7 Hz, 1 H) (imidazole-CH_2), and 7.12 (d, J = 1.0 Hz, 1 H) and 7.24 (d, J = 1.0 Hz, 1 H) (imidazole 4,5-H).

2-Nitro-α-[(2,2,3,3-tetramethyl-1-aziridinyl)methyl]-1*H*-imidazole-1-ethanol (6). 2,2,3,3-Tetramethylaziridine[14] [bp 102–103 °C (lit.[14] bp 104–104.5 °C); NMR ($CDCl_3$) δ ~1.28 (s, 1 H, NH) and 1.25 (s, 12 H, 4 × CH_3)] was treated with **7**, by the method previously described,[12] to give **6** (64%) as a pale yellow solid: mp 131–132 °C (lit.[12] mp 131–132 °C); NMR [$CDCl_3$ + $(CD_3)_2SO$; 1:1] δ 1.05 (s, 12 H, 4 × CH_3), 2.30 (d, J = 5 Hz, 2 H, aziridine-CH_2), 3.8 (br, 1 H, OH), 4.1–5.1 (m, 3 H, imidazole-CH_2C*H*), and 7.05 (br s, 1 H) and 7.50 (br s, 1 H) (imidazole 4,5-H).

1-(Oxiranylmethyl)-2-nitroimidazole (7). Alkylation of 2-nitroimidazole with 1-chloro-2,3-epoxypropane, followed by base-catalyzed ring closure, generally according to the method of Beaman et al.,[32] gave **7** (96%) as white needles: mp 55.5–56 °C (lit.[32] mp 53.5–55 °C); NMR ($CDCl_3$) δ 2.55 (dd, J = 5 Hz, J = 3 Hz, 1 H) and 2.90 (t, J = 5 Hz, 1 H) (CH_2O), 3.45 (ddt, J = 6 Hz, J = 5 Hz, J = 3 Hz, 1 H, CHO), 4.30 (dd, J = 14 Hz, J = 6 Hz, 1 H) and 5.08 (dd, J = 14 Hz, J = 3 Hz, 1 H) (imidazole-CH_2), and 7.10 (s, 1 H) and 7.25 (s, 1 H) (imidazole 4,5-H).

1-[(2,4-Dinitrophenyl)thio]-2,2,3,3-tetramethylaziridine (8). $Pb(OAc)_4$ (85%; 1.0 g, ca. 2 mmol) was added during 10 min to 2,4-dinitrobenzenesulfenamide[16] [mp 118–119 °C (lit.[15] mp 119–120 °C)] (430 mg, 2 mmol) and 2,3-dimethylbut-2-ene (840 mg, 10 mmol) in CH_2Cl_2 (3 mL). After a further 20 min, the solution was diluted with CH_2Cl_2 (10 mL) and was washed with saturated aqueous $NaHCO_3$. The organic phase was dried (Na_2SO_4) and filtered and the solvent was evaporated to afford **8** (550 mg, 93%) as an orange solid: mp 148–149 °C; IR (Nujol) 1590, 1510, and 1300 cm^{-1}; NMR ($CDCl_3$) δ 1.40 (s, 12 H, 4 × CH_3), 8.35 (dd, J = 9 Hz, J = 2 Hz, 1 H, 5-H), 8.45 (dd, J = 9 Hz, J = 0.5 Hz, 1 H, 6-H), and 9.06 (dd, J = 2 Hz, J = 0.5 Hz, 1 H, 3-H). Anal. ($C_{12}H_{15}N_3O_4S$) C, H, N.

α-[[(2-Chloroethyl)amino]methyl]-2-nitro-1*H*-imidazole-1-ethanol Hydrochloride (9). Method A. A solution of α-(aziridinylmethyl)-2-nitro-1*H*-imidazole-1-ethanol[8,11,12] (**3**; 2.12 g, 10 mmol) in acetone (20 mL) at 40 °C was cooled rapidly to 5–10 °C and was treated with a saturated solution of HCl in acetone (20 mL). After 2 min, decolorizing charcoal (300 mg) was added and the mixture was filtered while still warm from the exothermic reaction. On cooling of the filtrate, there was obtained **9** (2.66 g, 93%) as very pale yellow crystals: mp 153–154 °C dec. An analytical sample was recrystallized from aqueous acetone to give pale yellow prisms: mp 153–154 °C dec; IR (KBr) 3450 (br), 3345 (br), 3146, 3090, 1593, 1542, 1509, and 1495 cm^{-1}; NMR [$(CD_3)_2SO$] δ 3.1 (m, 2 H, CHC$H_2$$^+NH_2$), 3.4 [m (becomes t, J = 7 Hz, on decoupling at δ 9.35), 2 H, $^+NH_2CH_2CH_2Cl$], 3.95 (t, J = 7 Hz, 2 H, CH_2Cl), 4.2–4.7 (m, 4 H, imidazole-CH_2CHOH), 7.15 (d, J = 1 Hz, 1 H) and 7.65 (d, J = 1 Hz, 1 H) (imidazole 4,5-H), and 9.35 (br, 1 H, $^+NH_2$). Anal. ($C_8H_{14}Cl_2N_4O_3$) C, H, Cl, N.

α-[[(2-Chloroethyl)amino]methyl]-2-nitro-1*H*-imidazole-1-ethanol Hydrochloride (9). Method B. Aqueous HCl (10.5 M; 2 mL, 21 mmol) was added in one portion to a fine suspension of **3** (2.12 g, 10 mmol) in acetone (20 mL) at 0–5 °C. The solution was stirred for 5 min then treated with decolorizing charcoal (300 mg) and filtered. The filtrate was chilled to afford **9** (1.88 g, 66%) as pale yellow microcrystals: mp 152.5–153.5 °C dec, identical (mixed melting point, IR, analysis) with the material prepared by method A above.

α-[[(2-Bromoethyl)amino]methyl]-2-nitro-1*H*-imidazole-1-ethanol Hydrobromide (10). Treatment of **3** with anhydrous HBr, according to method A for the preparation of **9** above, gave **10** (96%) as very pale yellow crystals: mp 150.5–151.5 °C dec. Alternatively, treatment of **3** with 2 equiv of aqueous HBr (5.9 M), according to method B for the preparation of **9** above, except that the charcoal was omitted and the recrystallization solvent was aqueous acetone, gave **10** (74%) as pale yellow prisms: mp 150–151 °C dec; IR (KBr) 3450 (br), 3345 (br), 3160, 3140, 3087, 1590, 1539, 1507, and 1493 cm^{-1}; NMR [250 MHz; $(CD_3)_2SO$] 2.94 [m (+D_2O becomes dd, J = 12.6 Hz, J = 9.8 Hz), 1 H] and 3.15 [m (+D_2O becomes dd, J = 12.6 Hz, J = 2.7 Hz), 1 H] ($CHCH_2$$^+NH_2$), 3.46 [m (+$D_2O$ becomes t, J = 6.5 Hz, 2 H, $^+NH_2CH_2CH_2Br$], 3.68 (t, J = 6.5 Hz, 2 H, CH_2Br), 4.21 [m, (+D_2O becomes dddd, J = 9.8 Hz, J = 8.0 Hz, J = 3.9 Hz, J = 2.7 Hz), 1 H, C*H*OH], 4.31 (dd, J = 13.4 Hz, J = 8.0 Hz, 1 H) and 4.53 (dd, J = 13.4 Hz, J = 3.9 Hz, 1 H) (imidazole-CH_2), 5.95 (br d, J ≈ 5 Hz, 1 H, OH), 7.22 (s, 1 H) and 7.60 (s, 1 H) (imidazole 4,5-H), and 8.72 (br, 2 H, NH_2). Anal. ($C_8H_{14}Br_2N_4O_3$). From some preparations of **10** according to this method, a crystalline monohydrate (mp 161–162 °C) was obtained.

α-[[(2-Bromoethyl)amino]methyl]-2-nitro-1*H*-imidazole-1-ethanol Hydrobromide (10). Method C. In a modification of the general procedure of Furukawa et al.,[19] **22**[31] (2.30 g, 10 mmol) and dimethylbromosulfonium bromide[19] (2.22 g, 10 mmol) were stirred at 50 °C for 12 h in dry DMF (20 mL) before the solvent was evaporated. The residue, in ethanol (10 mL), was chromatographed (silica gel; $CHCl_3$, MeOH; 9:1). Treatment of the appropriate fraction of eluate with ethereal HBr gave **10** (680 mg, 18%) as a yellow solid: mp 148.5–150.5 °C dec, identical (mixed melting point, IR, analysis) with the material described above.

α-[[(2-Iodoethyl)amino]methyl]-2-nitro-1*H*-imidazole-1-ethanol Hydriodide (11). Treatment of **3** with 2 equiv of aqueous HI, according to method B for the preparation of **9** above, except that the recrystallization solvent was H_2O, gave **11** (51%) as yellow crystals: mp 172–173 °C dec; IR (KBr) 3460 (br), 3310 (br), 3135, 3106, 3092, 1539, 1509, and 1492 cm^{-1}; NMR [$(CD_3)_2SO$] δ 3.12 (m, 2 H, CHC$H_2$$^+NH_2$), 3.36 (s, 4 H), NCH_2CH_2I), 4.1–4.6 (m, 3 H, imidazole-CH_2CH), 5.8 (br, 1 H, OH), 7.16 (d, J = 1 Hz, 1 H) and 7.59 (d, J = 1 Hz, 1 H) (imidazole 4,5-H), and 8.6 (br, 2 H, $^+NH_2$). Anal. ($C_8H_{14}I_2N_4O_3$) H, N; C: calcd, 20.53; found 21.02; I: calcd, 54.23; found 53.76.

α-[[(2-Fluoroethyl)amino]methyl]-2-nitro-1*H*-imidazole-1-ethanol Hydrochloride (12). According to method B for the preparation of **9** above, **3** was treated with 2 equiv of aqueous HF (24 M; **CAUTION**). The reaction mixture was then stirred with ethanolic NaOH (5% w/w; 1.0 mol equiv) and decolorizing charcoal for 15 min. Treatment of the filtrate with a small excess of ethereal HCl gave **12** (41%) as a pale yellow solid: mp 177–179 °C dec, identical with the material described below.

α-[[(2-Fluoroethyl)amino]methyl]-2-nitro-1*H*-imidazole-1-ethanol Hydrochloride (12). Method D. Oxirane **7** (4.0 g, 23.7 mmol), (2-fluoroethyl)ammonium chloride (5.0 g, 50.2 mmol), and NaOH (2.0 g, 50 mmol) were stirred at 15 °C for 30 min in ethanol (60 mL) and heated to reflux for 3 h. The mixture was then treated with decolorizing charcoal (500 mg) and filtered. The evaporation residue, in ethanol (20 mL), was treated with a small excess of ethereal HCl to furnish, after recrystallization from aqueous ethanol, **12** (4.38 g, 69%) as pale yellow crystals: mp 178–179 °C dec; IR (KBr) 3450 (br), 3280 (br), 3140, 3090, 1580, 1531, and 1487 cm^{-1}; NMR [250 MHz; $(CD_3)_2SO$] δ 2.92 (m, (+D_2O becomes dd, J = 12.5 Hz, J = 9.9 Hz), 1 H] and 3.13 [m (+D_2O becomes dd, J = 12.5 Hz, J = 2 Hz), 1 H] ($CHCH_2$$^+NH_2$), 3.33 [br d, J = 27.9 Hz (+D_2O becomes dt, J = 27.9 Hz, J = 6.5 Hz), 2 H, $^+NH_2CH_2CH_2F$], 4.25 (m, 1 H, C*H*OH), 4.39 (dd, J = 13.6 Hz, J = 7.8 Hz, 1 H) and 4.58 (dd, J = 13.4 Hz, J = 4.0 Hz, 1 H) (imidazole-CH_2), 4.76 (dt, J = 47.1 Hz, J = 4.4 Hz, 2 H, CH_2F), 6.04 (br, 1 H, OH), 7.20 (s, 1 H) and 7.66 (s, 1 H) (imidazole 4,5-H), and 9.14 (br, 1 H) and 9.28 (br 1 H) NH_2. Anal. ($C_8H_{14}ClFN_4O_3$) C, H, Cl, F, N.

α-[[(*threo*-2-Chloro-1-methylpropyl)amino]methyl]-2-nitro-1*H*-imidazole-1-ethanol Hydrochloride (13). Compound **4** was treated with aqueous HCl generally according to method B for the preparation of **9** above, except that the charcoal was omitted and the recrystallization solvent was ethanol, to give **13** (88%) as a very pale yellow solid: mp 138.5–139 °C dec; NMR (300 MHz; $(CD_3)_2SO$) δ 1.30 (d, J = 6.6 Hz, 1.5 H, NCHCH_3 of one diastereoisomer), 1.31 (d, J = 6.7 Hz, 1.5 H, NCHCH_3 of other

(32) Beaman, A. G.; Tautz, W.; Duschinsky, R. *Antimicrob. Agents Chemother.* 1986, 520.

diastereoisomer), 1.52 (d, J = 6.7 Hz, 3 H, ClCHCH_3), 2.96 (m, 1 H) and 3.16 (m, 1 H) and 3.52 (m, 0.5 H) and 3.52 (m, 0.5 H) (C$H_2$$^+$$NH_2CH$), 4.27 (m, 1 H) and 4.39 (m, 1 H) and 4.57 (m, 3 H) (imidazole-CH_2CHOH and ClCH), 7.19 (br s, 1 H) and 7.65 (br s, 1 H) (imidazole 4,5-H), and 8.70 (br, 0.5 H) and 8.84 (br, 0.5 H) and 9.02 (br, 0.5 H) and 9.38 (br, 0.5 H) ($^+NH_2$). Anal. ($C_{10}H_{18}Cl_2N_4O_3$) C, H, Cl, N.

α-[[(*threo*-2-Bromo-1-methylpropyl)amino]methyl]-2-nitro-1*H*-imidazole-1-ethanol Hydrobromide (14). Aziridine **4** was treated with aqueous HBr generally according to method B for the preparation of **9** above, except that the charcoal was omitted, to give **14** (83%) as a pale yellow powder: mp 165–166 °C dec; NMR [D_2O + CF_3CO_2D (8:1)] δ 1.50 (d, J = 7 Hz, (becomes s on decoupling at δ 3.65), 3 H, NCHCH_3], 1.85 [d, J = 6 Hz, (becomes s on decoupling at δ 4.4), 3 H, BrCHCH_3], 3.5 (m, 2 H, C$H_2$$^+$$ND_2$), 3.65 (m, 1 H, NC$HCH_3$), 4.3–4.8 (m, 4 H, imidazole-CH_2CH and BrCH), and 7.30 (br s, 1 H) and 7.55 (br s, 1 H) (imidazole 4,5-H). Anal. ($C_{10}H_{18}Br_2N_4O_3$) C, H, N.

α-[[(2-Chloro-1,1-dimethylethyl)amino]methyl]-2-nitro-1*H*-imidazole-1-ethanol Hydrochloride (15). α-[(2,2-Dimethyl-1-aziridine)methyl]-2-nitro-1*H*-imidazole-1-ethanol[8,11,12] (**5**) was treated with aqueous HCl, generally according to method B for the preparation of **9** above, except that the reaction was carried out at 40 °C and the recrystallization solvent was aqueous acetone containing a trace of HCl, to yield **15** (78%) as colorless prisms: mp 196.5–198 °C dec; NMR [D_2O + CF_3CO_2D (8:1)] δ 1.60 (s, 6 H, 2 × CH_3), 3.35 (dd, J = 13 Hz, J = 2 Hz, 1 H) and 3.42 (dd, J = 13 Hz, J = 4 Hz, 1 H) ($CH_2$$^+$$ND_2$), 3.92 (s, 2 H, CH_2Cl), 4.4–5.0 (m, 3 H, imidazole-CH_2CH), and 7.30 (br s, 1 H) and 7.53 (br s, 1 H) (imidazole 4,5-H). Anal. ($C_{10}H_{18}Cl_2N_4O_3$) C, H, Cl, N.

α-[[(2-Bromo-1,1-dimethylethyl)amino]methyl]-2-nitro-1*H*-imidazole-1-ethanol Hydrobromide (16). Aziridine **5**[8,11,12] was treated with aqueous HBr generally according to method B for the preparation of **9** above, except that the reaction was carried out at 40 °C, to yield **16** (82%) as very pale yellow prisms: mp 186–197 °C dec; NMR [$(CD_3)_2SO$] δ 1.42 (s, 6 H, 2 × CH_3), 3.1 (m, 2 H, C$H_2$$^+$$NH_2$), 3.80 (s, 2 H, CH_2Br), 4.1–4.6 (m, 3 H, imidazole-CH_2CH), 5.5 (br, 1 H, OH), 7.12 (d, J = 1 Hz, 1 H) and 7.53 (d, J = 1 Hz, 1 H) (imidazole 4,5-H), and 8.6 (br, 2 H, $^+NH_2$). Anal. ($C_{10}H_{18}Br_2N_4O_3$) C, H, N; Br: calcd, 39.75; found 39.23.

α-[[(2-Bromo-1,1-dimethylethyl)amino]methyl]-2-nitro-1*H*-imidazole-1-ethanol Hydrobromide (16). Method E. In a modification of the method of Ponpipom and Hanessian,[20] **19** (2.58 g, 10 mmol), *N*-bromosuccinimide (NBS; 2.67 g, 15 mmol), and triphenylphosphine (3.93 g, 15 mmol) were stirred in dry DMF (50 mL) at 50 °C for 1.5 h. The solvent was evaporated and the residue was extracted with ethanol (20 mL). This solution was filtered. Column chromatography (silica gel; $CHCl_3$, MeOH; 9:1) and treatment of the appropriate fraction of eluate with a small excess of ethereal HBr gave **16** (1.09 g, 27%) as a yellow solid: mp 181–184 °C dec. Anal. ($C_{10}H_{18}Br_2N_4O_3$) C, H, N; Br: calcd 39.75; found 38.91. TLC analysis indicated the presence of a very slight trace of triphenylphosphine oxide.

α-[[(2-Chloro-1,2,2-trimethylpropyl)amino]methyl]-2-nitro-1*H*-imidazole-1-ethanol Hydrochloride (17). Aziridine **6** was treated with aqueous HCl generally according to method B for the preparation of **9** above, except that the charcoal was omitted and the reaction was carried out at 20 °C, to give **17** (88%) as colorless microcrystals: mp 183–184 °C dec; NMR [250 MHz; $(CD_3)_2SO$] δ 1.42 (s, 3 H) and 1.44 (s, 3 H) (NC$(CH_3)_2$), 1.73 (s, 3 H) and 1.74 (s, 3 H) [C$(CH_3)_2$Cl], 3.11 [m, (^+D_2O becomes dd, J = 12.5 Hz, J = 9.9 Hz), 1 H] and 3.33 [m, (^+D_2O becomes brd, $J \approx$ 12 Hz), 1 H] ($^+NH_2$CH_2), 4.32 [m, (^+D_2O becomes ddd, J = 9.9 Hz, J = 8.1 Hz, J = 3.7 Hz), 1 H CHOH], 4.42 (dd, J = 13.3 Hz, J = 8.1 Hz, 1 H) and 4.61 (dd, J = 13.3 Hz, J = 3.7 Hz, 1 H) (imidazole-CH_2), 6.25 (br, 1 H, OH), 7.21 (d, J = 0.8 Hz, 1 H) and 7.66 (d, J = 0.8 Hz, 1 H) (imidazole 4,5-H), and 7.97 (br quintet, $J \approx$ 7 Hz, 1 H) and 9.10 (br, 1 H) ($^+NH_2$). Anal. ($C_{12}H_{22}Cl_2N_4O_3$) C, H, N.

α-[[(2-Bromo-1,2,2-trimethylpropyl)amino]methyl]-2-nitro-1*H*-imidazole-1-ethanol Hydrobromide (18). Aziridine **6** (810 mg, 3 mmol), in acetone (10 mL), was treated with aqueous HBr (6 M; 1.2 mL, 7.2 mmol) at reflux for 10 min before being cooled. Recrystallization of the evaporation residue from aqueous acetone afforded **18** (950 mg, 73%) as pale yellow crystals: mp 163–164 °C dec; NMR ($(CD_3)_2SO$) δ 1.45 (s, 6 H, 2 × CH_3), 1.50 (s, 6 H, 2 × CH_3), 3.2 (m, 2 H, $^+NH_2$CH_2), 4.2–4.7 (m, 3 H, imidazole-CH_2CHOH), 5.2 (br, 1 H, OH), 7.15 (br s, 1 H) and 7.65 (br s, 1 H) (imidazole 4,5-H), and 8.1 (br, 1 H) and 8.7 (br, 1 H) ($^+NH_2$). Anal. ($C_{12}H_{22}Br_2N_4O_3$) C, H, Br, N.

α-[[(2-Hydroxy-1,1-dimethylethyl)amino]methyl]-2-nitro-1*H*-imidazole-1-ethanol (19). Oxirane **7** (4.0 g, 23.7 mmol) and 2-amino-2-methylpropanol (10.0 g, 112 mmol) were boiled under reflux in ethanol (100 mL) for 45 min. The solvent was evaporated and the residue was extracted with hot ether (75 mL). The extract was cooled to 0 °C and the precipitated solid was recrystallized from ethanol to furnish **19** (4.94 g, 81%) as almost colorless prisms: mp 114.5–115.5 °C; NMR [D_2O + CF_3CO_2D (8:1)] δ 1.55 (s, 6 H, 2 × CH_3), 3.2 (dd, J = 13 Hz, J = 2 Hz, 1 H) and 3.55 (dd, J = 13 Hz, J = 4 Hz, 1 H) ($^+ND_2$CH_2), 3.85 (s, 2 H, CH_2O), 4.3–5.0 (m, 3 H, imidazole-CH_2CH), and 7.40 (d, J = 1 Hz, 1 H) and 7.65 (d, J = 1 Hz, 1 H), (imidazole 4,5-H). Anal. ($C_{10}H_{18}N_4O_4$) C, H, N.

α-[(Ethylamino)methyl]-2-nitro-1*H*-imidazole-1-ethanol Hydrochloride (21). Oxirane **7** (507 mg, 3 mmol) was boiled under reflux with 70% aqueous $EtNH_2$ (2 mL) in ethanol (8 mL) for 1 h. The solvents and excess reagent were evaporated. Dry ethanol (20 mL) was added and was subsequently evaporated. The residue, in dry ethanol (4 mL), was treated with ethereal HCl (1 M, 4.5 mL, 4.5 mmol). The precipitate recrystallized from ethanol to furnish **21** (650 mg, 87%) as pale yellow crystals: mp 196–197 °C; IR (Nujol) 3300 (br), 1540, 1505, and 1495 cm^{-1}; NMR ($(CD_3)_2SO$) δ 1.20 [t, J = 7 Hz (becomes s on decoupling at δ 3.00), 3 H, CH_3], 3.7–3.1 (m, 4 H, C$H_2$$^+$$NH_2CH_2$), 4.2–4.6 (m, 3 H, imidazole-CH_2CH), 5.9 (br, 1 H, OH), 7.10 (d, J = 1 Hz, 1 H) and 7.58 (d, J = 1 Hz, 1 H) (imidazole 4,5-H), and 8.8 (br, 2 H, $^+NH_2$). Anal. ($C_8H_{15}ClN_4O_3$) C, H, Cl, N.

HPLC Analysis for Stability of Drugs. The stability of compounds **9–11** and **13–18** was determined at 4 °C and at 23 °C by incubation of the agents (0.2–0.5 mM) in aqueous solution containing phosphate-buffered saline (PBS, Oxoid) and in the presence or absence of sodium hydrogen carbonate (24–107 mM) at pH 5–8. In all cases, the pH of the solutions was adjusted immediately following their preparation. At given times, aliquots were assayed for degradation of the compounds with HPLC. A Waters HPLC system with a UV–vis detector set at 313 nm (Model 440) was used to perform isocratic elutions with a CN column (Waters Resolve CN, 10 μm or a Nova-Pak CNHP, 4 μm). The mobile phase (flow rate 2 mL min^{-1}) contained 10–25% methanol in aqueous KH_2PO_4 (10 mM) at pH 3.0 (see Table I). The rate of release of Br^- from **10** in PBS solution was determined with a bromide ion selective electrode (Radiometer F1022Br) in conjunction with a pH meter (Radiometer PHM64).

Biological Studies. In Vitro. Chinese hamster V79-379A cells, maintained in suspension culture, were plated onto 6 cm diameter glass Petri dishes in MEM (modified essential medium) containing 10% fetal calf serum and allowed to attach for 2 h. For radiosensitization studies, the medium was then replaced with 2 mL of a freshly prepared solution of the agent in PBS at pH 7.4, and the Petri dishes were placed into gas-tight dural vessels. Hypoxia was induced by purging the vessels with N_2 (<10 ppm O_2) for 1 h prior to irradiation at 22 °C with ^{60}Co γ-rays at a dose rate of 8 Gy min^{-1}. The solution of drug was removed after irradiation and replaced with fresh medium. The cells were incubated at 37 °C for 7 days before assaying for formation of colonies. Sensitizer enhancement ratios were determined from the ratio of slopes of survival curves for hypoxic conditions in the presence and absence of the agent. For cytotoxicity assays, cells were plated into 100-mL glass bottles (1.5 × 10^6 cells/bottle), allowed to grow to confluency over 48 h and exposed to various concentrations of the agents for 3 h in growth medium under either hypoxic or aerobic conditions at 37 °C. Cultures were then trypsinized, diluted, and plated to assay formation of colonies.

Biological Studies. In Vivo. C3H/He mice were bred at the Radiobiology Unit to provide U.K. specified pathogen free, category IV, 8–12 week old female mice for experiments. The KHT sarcoma[33] was provided by Dr. P. Twentyman, MRC

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Cambridge, U.K., in 1983 and maintained by intramuscular inoculation of a tumor brei for up to 12 consecutive passages and then reestablished from frozen stocks. For experimentation, 2–4 $\times 10^5$ viable tumor cells were injected subcutaneously in the middorsal pelvic region of the mice. Treatments were initiated 10–14 days later when tumors reached a maximum diameter of 6–8 mm.

Prior to injection, compounds were usually dissolved at 20 °C in PBS at pH 7.0 by means of sonication for up to 5 min. Compounds **9** and **10** were dissolved in phosphate buffer at pH 5 to minimize cyclization to **3**. All solutions were administered within 10 min of preparation. Solutions were administered by the intraperitoneal route in a volume of 0.5 mL per 25 g of mouse body weight, with the exception of **14**, **16**, and **18**, which were administered in a volume of 1 mL per 25 g owing to their dose-limiting solubility. Escalating doses of each of the test compounds were administered to groups of two or three tumor-bearing mice to establish the maximum tolerated dose (MTD). The MTD is defined as the highest single dose which does not produce severe or persistent clinical signs or death of the animals within 24 h.

The responses of KHT sarcomas to therapy administered in vivo were measured by means of a soft agar clonogenic assay in vitro,[34] as described recently.[35] Tumors were excised 18–24 h after treatment and each was assayed individually. Surviving fractions were calculated as the ratios of the numbers of colonies scored to the number of viable cells plated, relative to the plating efficiency of a control tumor processed at the same time. The mean plating efficiency of cells from 66 untreated tumors was 49.5 ± 1.8% (mean ± SE) and the yield of cells ranged between 8.9 $\times 10^6$ and 7.6 $\times 10^7$ cells g^{-1} of tumor tissue. A Pantac X-ray set was used to produce 250 kV X-rays (15 mA) at a dose rate of 3.8 Gy min^{-1} with a half-value layer (HVL) equivalent to 1.33 mm Cu. Radiation doses were monitored with an air chamber corrected for ambient temperature and pressure. Unanesthetized mice were restrained in polyvinyl jigs with Pb shielding and a cutaway section to allow local irradiation of tumor by the unilateral beam.[36] A dose of 10 Gy of X-rays was used to test the efficiency of each compound as a hypoxic cell radiosensitizer. Each experiment included mice exposed to a 10 Gy dose of X-rays without drug and mice treated with drug without radiation. There is a minimum of three tumors in each group; most points were determined from the geometric mean of surviving fractions from at least four to six tumors from two separate experiments. Initially, the optimum time of administration before irradiation for maximum radiosensitization was determined for each of the compounds by administering the MTD. Subsequently a range of single doses of the compounds were administered at the optimum time (45–60 min in each case) before exposure to X-rays to obtain a drug dose/response curve for radiosensitization. Where appropriate, direct comparisons of radiosensitization by equimolar doses of chemically related compounds were made in a single experiment.

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PUBLICATION 40

Selective Reductions of 1-(Carbonyl)-Substituted 2-Nitroimidazoles with Alkali Metal Borohydrides and Borane.Tetrahydrofuran

M. D. Threadgill and P. Webb

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SELECTIVE REDUCTIONS OF 1-(CARBONYL)SUBSTITUTED 2-NITRO-IMIDAZOLES WITH ALKALI METAL BOROHYDRIDES AND WITH BORANE.TETRAHYDROFURAN

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Abstract: Sodium borohydride, lithium borohydride and borane.THF reduce carbonyl groups (esters, amide) in 1-substituted 2-nitroimidazoles in high yield without reduction of the nitroheterocycle.

1-Substituted 2-nitroimidazoles sensitise[1-4] hypoxic cells to killing by ionising radiation by acting as electron-affinic mimics of dioxygen and are reduced readily by biological systems[5] to substances which can be selectively retained and be cytotoxic towards cells deficient in dioxygen[6]. During our programme of synthesis of substituted 2-nitroimidazoles, selective reductions of various types of carbonyl group have been required while retaining the nitroheterocycle (E^1_7 = *ca.* -380 mV)[7]. We have already demonstrated reductions of ketones and aldehydes attached to nitroimidazoles by sodium borohydride under very mild conditions[8,9]; the reductions addressed here are of esters to the corresponding alcohols and of a primary amide to a primary amine.

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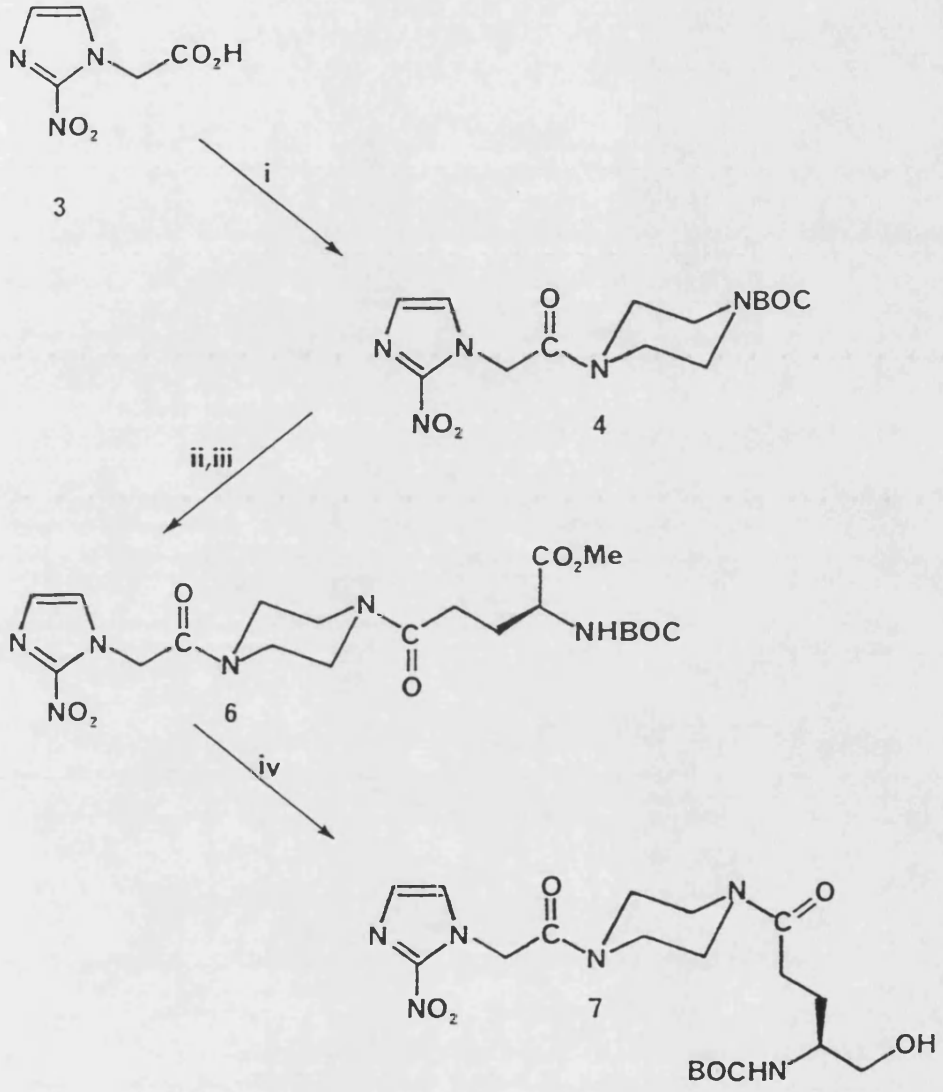
SCHEME 2



Reagents: (i) 1-BOCpiperazine/Dicyclohexylcarbodiimide/THF; (ii) CF_3CO_2H; (iii) Glutamic acid α-methyl ester γ-(4-nitrophenyl) ester (S)/diisopropylethylamine/THF; (iv) $LiBH_4$/THF.

of substituted 2-nitroimidazoles with different physico-chemical and pharmacokinetic parameters for therapeutic and diagnostic applications, particularly facilitating isotopic labelling.

EXPERIMENTAL

IR, NMR and mass spectra were obtained using Philips PU9516, Jeol PMX60SI and VG7070 spectrometers, respectively. Melting points are uncorrected. Tetrahydrofuran (THF) was distilled from CaH_2 before use. Radial PLC refers to centrifugally accelerated preparative layer chromatography performed using a Chromatotron (TC Research). Solvents were evaporated under reduced pressure.

2-Nitroimidazole-1-ethanol (2).- Methyl 2-nitroimidazole-1-acetate[14] (1) (580 mg, 3.14 mmol) was stirred with $NaBH_4$ (630 mg, 16.6 mmol) in EtOH (20 mL) for 1.5 h. Aqueous HCl (9 M, 2 mL) was added carefully. The suspension was filtered and the solvents were evaporated from the combined filtrate and MeOH washings. Column chromatography (silica gel, $CHCl_3$:MeOH, 9:1) afforded (2) (440 mg, 89%) as an off-white solid: mp 113.5-115°C (lit.[19] mp 116°C). δ ($CDCl_3$ + CD_3OD, 2:1) 3.95 (2 H, t, *J* 5 Hz, CH_2OD), 4.65 (2 H, t, *J* 5 Hz, imidazole-CH_2), 7.15 (s, 1 H, imidazole 4-H), 7.45 (s, 1 H, imidazole 5-H).

1-(*t*-Butoxycarbonyl)-4-(2-nitroimidazol-1-ylacetyl)piperazine (4).- 2-Nitroimidazole-1-acetic acid (3)[3] (513 mg, 3 mmol) was stirred with dicyclohexylcarbodiimide (700 mg, 3.4 mmol) and 1-(t-butoxycarbonyl)piperazine[11] (558 mg, 3 mmol) in THF (13 mL) for 24 h. The suspension was filtered and the solvents were evaporated from the combined filtrate and Et_2O washings. Radial PLC (silica gel, CH_2Cl_2) gave (4) (848 mg, 83%) as a glass: δ ($CDCl_3$) 1.45 (9 H, s, Bu^t), 3.55 (8 H, s, piperazine-H), 5.30 (2 H, s, imidazole-CH_2); 7.10 (2 H, s, imidazole 4,5-H); *m/z* (EI) 339.1533 (M^+) ($C_{14}H_{21}N_5O_5$ requires 339.1543).

1-(*S*-4-(*t*-Butoxycarbonylamino)-5-methoxy-5-oxopentanoyl)-4-(2-nitroimidazol-1-ylacetyl)piperazine (6).- Protected piperazine (4) (848 mg, 2.5 mmol) was stirred with trifluoroacetic

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PUBLICATION 41

Dimethylacetamide

A. Gescher and M. D. Threadgill

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Vol. 2, 2nd Edition

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[Book chapter]

2.1. *N,N*-Dimethylacetamide

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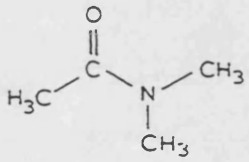
Synonyms: Acetamide, Dimethyl; *N,N*-Dimethylacetamide; DMA

CAS-Number: 127-19-5

Structural formula:



Molecular formula: C_4H_9NO

Molecular weight: 87.12

Properties:

boiling point: 166.1°C
melting point: −20°C
flash point: 70°C (158°F)
vapor pressure: 0.27 kPa (2 mmHg) at 25°C
vapor density (air = 1.00): 2.97
specific gravity (liquid density): 0.9366 (25°C/4°C)
refractive index: 1.4375 at 20°C (sodium D line)
conversion factors (at 25°C): 1 p.p.m. = 3.563 mg/m^3
1 mg/m^3 = 0.2806 p.p.m.
solubility: miscible with ethanol, ether, acetone, water, chloroform, benzene, methanol

Ethel Browning's Toxicity and Metabolism of Industrial Solvents, 2nd edition.
Vol. 2: Nitrogen and Phosphorus Solvents. D.R. Buhler and D.J. Reed, editors

A. Gescher and M.D. Threadgill

ECONOMY, SOURCES AND USES

PRODUCTION

Dimethylacetamide has been produced by the reaction of acetic acid with dimethylamine (Siegle, 1980).

INDUSTRIAL USES

Dimethylacetamide is a powerful industrial solvent, the uses of which are very similar to those of dimethylformamide (Siegle, 1980). Its strong solvent action renders it particularly useful in the manufacture of films and fibers and as a solvent for polyacrylonitrile, polyvinyl chloride, polyamides, cellulose derivatives and polystyrenes and in coatings and adhesive formulations. Dimethylacetamide dissolves many inorganic salts.

BIOCHEMISTRY

ESTIMATION

Gas chromatography seems to be the method of choice for the analytical determination of dimethylacetamide.

METABOLISM

Gas chromatographic analysis of the urine of rats which had received dimethylacetamide by the subcutaneous route indicated the presence of *N*-methyl-acetamide and acetamide (Barnes and Ranta, 1972). Both metabolites were also found by the same authors in incubation mixtures of dimethylacetamide with rat liver homogenate. *N*-Methylacetamide was detected in the urine of human volunteers who had inhaled dimethylacetamide or absorbed dimethylacetamide vapor through the skin (Maxfield et al 1975). Measurement of the amount of the metabolite *N*-methylacetamide excreted by individuals exposed to dimethylacetamide vapors with or without face masks which allowed the inhalation of air free of dimethylacetamide indicated that more dimethylacetamide was absorbed through the lungs than through the skin. Interestingly, in this study, only 2–10% of the amount of dimethylacetamide inhaled was recovered in the urine in the form of *N*-methylacetamide. It has been suggested that the major urinary metabolite of the analogous dimethylformamide is *N*-(hydroxymethyl)-*N*-methylformamide and not N-methylformamide, since the carbinolamide decomposes on the gas chroma-

tography column (to *N*-methylformamide) but is relatively stable in aqueous solution (Kestell et al 1986). In analogy, it would be logical to assume that the *N*-methylacetamide found in the urine after exposure to dimethylacetamide really arose from chemical breakdown of *N*-(hydroxymethyl)-*N*-methylacetamide during the analytical process. This contention, however, remains to be proven.

TOXICOLOGY

Dimethylacetamide is an agent of only moderate toxic potential. Its major target organ of toxicity appears to be the liver. Dimethylacetamide is also embryotoxic.

ANIMAL TOXICITY

Acute

Lethal dose. The LD_{50} of a single dose of dimethylacetamide ranged between 2 and 5 g/kg in rats and mice, depending on the route of administration (Bartsch et al 1976; Caujolle et al 1970; Davis and Jenner, 1959; Ochia, 1980; Smyth et al 1962; Thiersch, 1962; Wiles and Narcisse, 1971). The oral LD_{50} in male and female rats was found to be similar, at 5.8 and 4.9 g/kg respectively (Kennedy and Sherman, 1986). The 1 hour LC_{50} by inhalation in this species was 2475 p.p.m. (Kennedy and Sherman, 1986).

Chronic

Repeated administration of 0.5 g/kg orally to rats daily for 1 month did not cause pathological lesions detectable by microscopy (Auclair and Hameau, 1964). At 1.9 g/kg applied dermally to rats daily on 10 consecutive days, death occurred between the sixth and tenth dose (Stula and Kraus, 1977); in this study, severe inflammation and irritation of the stomach and the lungs was recorded. Cutaneous application of dimethylacetamide at 4 g/kg/day for 5 days/week for 6 weeks killed dogs after they had experienced weakness, ataxia, diarrhea, loss of weight and jaundice (Horn, 1961). Hepatic damage was indicated by increases in the bromosulphothalein retention time and the activity of serum alkaline phosphatase and was confirmed by histopathology. In contrast, the dogs survived this treatment for 6 months when the dose was only 1 g/kg and toxicity was not observed except for pale coloration and fatty degeneration in the livers. The central nervous system of rabbits which received 0.25 to 2 g/kg given by different routes for 21 days or longer was affected as evidenced by variable electroencephalographic changes, dysrhythmias and electrographic seizures at the higher doses (Steiner and

Himwich 1964). In this species, dimethylacetamide caused mild irritation when applied undiluted to the belly (Smyth et al 1962).

Hepatotoxicity

One month after intraperitoneal administration of a near lethal dose of dimethylacetamide to mice, damage to hepatocytes was observed together with necrotic pancreatitis and severe necrosis of splenic lymphocytes (Caujolle et al 1970). In this study, testicular atrophy was also seen four days post treatment. Post mortem examination of rats which had received the LD_{50} of dimethylacetamide via the oral route revealed generalized hemorrhage in several organs and necrosis in the liver and kidneys (Kafyan 1971). Dermal doses of 2 g/kg caused death of rabbits from acute hepatic necrosis (Kennedy and Sherman 1986).

Reproductive toxicity

Dimethylacetamide was embryotoxic in rats when a single dose of 2 g/kg was administered via the intraperitoneal route on days 4 and 14 of gestation. This dose produced the resorption of all litters (Thiersch 1962). Daily oral administration of dimethylacetamide (0.4 g/kg) to rats from day 6 to day 19 of gestation caused fetal malformations accompanied by toxicity to the maternal organism (Johanssen et al 1985). Reproduction in rats was not influenced by repeated exposure to dimethylacetamide vapors up to 300 p.p.m. (Ferenz and Kennedy 1986). In rabbits, daily oral doses of 0.09 g/kg from day 6 to 19 of gestation had no adverse effect; at 0.29 g/kg fetal resorption was observed, together with weight loss in the mothers. Repeated administration of 0.47 g/kg was very toxic to the mothers and caused total resorption in the surviving animals (Merkle and Zeller, 1980).

HUMAN TOXICITY

A study of 41 workers who had been exposed to dimethylacetamide from 2 to 10 years revealed the occurrence of disorders reflecting liver damage (Corsi, 1971). Retention of bromosulfophthalein was increased in 9 of 10 workers who had been exposed to dimethylacetamide for 7 to 10 years, and in 10 of 20 workers who had been exposed to dimethylacetamide for 2 to 7 years. Other parameters of hepatic function which were altered in the exposed individuals include proteinemia, cholesterolemia, activities of hepatic transaminases and alkaline phosphatase in serum, and bilirubinemia. Hepatomegaly was diagnosed in 14 workers.

In a clinical trial, dimethylacetamide was administered to patients with advanced malignancies and caused abnormal mental states (Weiss et al 1962a, b). This effect was observed at a dose of 400 mg/kg given daily for 3 or more days.

The symptoms were not seen at 300 mg/kg or lower. The second or third dose caused depression, lethargy, occasional confusion or disorientation. The fourth or fifth dose produced striking hallucinations, perceptual distortions and delusions in all nine patients. All patients reverted to normal several days after discontinuation of treatment with dimethylacetamide.

GENETIC TOXICITY

Dimethylacetamide was not mutagenic in a number of tests *in vitro* (Kennedy 1986; McGregor 1980). Nor was there an increase in the incidence of tumors when dimethylacetamide was given orally to rats at either 0.1, 3, 10 or 30 mg/kg (Hadidian et al 1968).

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PUBLICATION 42

N-Methylacetamide

A. Gescher and M. D. Threadgill

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Vol. 2, 2nd Edition

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[Book chapter]

2.4. *N*-Methylacetamide

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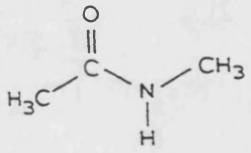
Pharmaceutical Sciences Institute, Aston University, Aston Triangle, Birmingham, B4 7ET, UK

Synonyms: Monomethylacetamide; Acetamide, N-Methyl; MMAC

CAS-Number: 79-16-3

Structural formula:



Molecular formula: C_3H_7NO

Molecular weight: 73.10

Properties:

boiling point: 1204–206°C
melting point: 28°C
flash point: 108°C
vapor pressure: 12.3 Pa (0.92 mmHg) at 25°C
vapor density: (air = 1.00): 2.49
specific gravity (liquid density): 0.9571 at 25°C/4°C
refractive index: 1.4301 at 20°C (sodium D line)
conversion factors (at 25°C): 1 p.p.m. = 0.3345 mg/m^3
1 mg/m^3 = 2.990 p.p.m
solubility: miscible with ethanol, ether, acetone, water, chloroform, benzene

Ethel Browning's Toxicity and Metabolism of Industrial Solvents. 2nd edition.
Vol. 2: Nitrogen and Phosphorus Solvents. D.R. Buhler and D.J. Reed, editors

ECONOMY, SOURCES AND USES

PRODUCTION

N-Methylacetamide has been prepared by reaction of methylamine with hot acetic acid (D'Alelio and Reid, 1937) and with acetic anhydride (Mauger and Soper, 1946). Other methods include heating *N,N'*-dimethylurea with acetic acid (US Patent, 1936) and reduction/hydrogenation of *N*-(hydroxymethyl)acetamide (US Patent, 1944).

INDUSTRIAL USES

Even though *N*-methylacetamide shares many general physical and chemical properties with dimethylacetamide, it has not found the extensive industrial applications of the latter. *N*-methylacetamide dissolves many inorganic salts.

BIOCHEMISTRY

ESTIMATION

The method of choice for the detection and estimation of *N*-methylacetamide appears to be gas chromatography (Barnes and Henry, 1974).

METABOLISM

In a recent comparative toxicity and metabolism study on four formamides and on *N*-methylacetamide, the sole metabolite of *N*-methylacetamide in the urine of mice was identified as *N*-(hydroxymethyl)acetamide (Kestell et al 1987). There was no evidence of induction of hepatic drug metabolizing enzymes in rats following treatment with *N*-methylacetamide (Ackerman and Leibman, 1977). *N*-Methylacetamide influenced neither the sleeping time induced by hexobarbital nor the metabolism of hexobarbital or aniline.

TOXICOLOGY

N-methylacetamide is a diabetogen and is embryotoxic.

ANIMAL TOXICITY

Acute

Lethal dose. The LD_{50} values for *N*-methylacetamide are between 5 and 7 g/kg in the rat after intraperitoneal, subcutaneous or oral administration (Caujolle et al 1970; Peters et al 1966; Thiersch, 1962) and 6.1 g/kg after intraperitoneal administration in the mouse (Caujolle et al 1970).

Diabetogenicity and other toxicities

Repeated oral dose of 0.8 g/kg per day for an unspecified period caused weight loss in rats and an increase in fasted blood sugar concentrations whereas glucose tolerance was decreased (Peters et al 1966). There was also evidence of pathological change in pancreatic tissue. A single subcutaneous dose of 10 g/kg impaired metabolism of carbohydrates in rats which led to an increased elimination of glucose (Sitt et al 1966). The same workers observed an increase in the secretion of glucorticoids and epinephrine and a decrease in inactivation of glucocorticoids, an increase in glomerular filtration rate and an enhanced secretion of insulin following intravenous administration of *N*-methylacetamide. Furthermore, the efficacy of endogenous or exogenous insulin was reduced. *N*-Methylacetamide is diabetogenic in the rat (Guidoux and Peters, 1965; Guidoux, 1969). An oral dose of 3.5 g/kg was diabetogenic in all animals, lower doses causing reversible hyperglycemia which lasted for several days. At 0.8 g/kg or less administered orally, *N*-methylacetamide showed no toxicity or diabetogenicity.

Reproductive toxicity

The embryotoxicity of *N*-methylacetamide was shown in rats after intraperitoneal administration of a single dose of or exceeding 1 g/kg which caused resorption of all fetuses (Thiersch, 1962). Repeated dermal application of *N*-methylacetamide (0.6 g/kg) to rabbits on days 9–11 or 11 and 12 or 12 and 13 of gestation produced only a slight increase in the fetal resorption (Stula and Kraus, 1977). The repeated daily oral administration of 0.24 g/kg on gestation days 6 through 18 produced fetal malformations as well as maternal toxicity in this species (Merkle and Zeller, 1980). When 0.05 g/kg was administered in this study, five malformed kits were observed from three litters without maternal toxicity; 0.01 g/kg was without adverse effects.

GENETIC TOXICITY

N-Methylacetamide caused a small but consistent increase in the number of

revertants in a bacterial mutagenicity test using *E. coli* at 10, 20 or 50 mg/plate (Hemmerly and Demerec, 1955).

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US PATENT 2109941 (1936).

US PATENT 2422400 (1944).

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The Chemistry of Azolotetrazinones

M. D. Threadgill

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[Book chapter]

6 The chemistry of azolotetrazinones

M. D. THREADGILL

6.1 Introduction

In 1978, it was believed[1,2] that the 1,2,3,5-tetrazine ring was inherently unstable, despite claims[3] of the preparation of this system. Indeed, there is only one confident report[4] of a [2,5-dihydro]-1,2,3,5-tetrazine without fusion to an azole. In 1979, Ege[5] published the first report of pyrazolo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-ones. There followed comprehensive reports[6-8] by the Aston group of the synthesis and antitumour activity of 3-alkyl and 3-aryl-8-substitutedimidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-ones and an indazolotetrazinone. 6-Alkylimidazotetrazinones and pyrazolotetrazinones have also been prepared by this group[9]. A group from the University of Kansas and from Warner Lambert/Parke-Davis have described[10] a further two pyrazolo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-ones closely analogous to the active lead compounds of Stevens *et al.*[7] The Heidelberg group followed up their first report of the ring system with an extensive study[11] of the synthesis of some forty pyrazolo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-ones, together with some 3-aryltriazolotetrazinones, by three distinct routes. Pyrrolo- and tetrazolo-tetrazinones remain unknown. The ring structures and numbering system (according to the IUPAC convention) of the two series of azolotetrazinones (1, 2) are shown in Figure 6.1.

Extensive studies have been carried out on the antitumour and other biological activities of the imidazotetrazinones. 8-Carbamoyl-3-(2-chloroethyl)imidazo-[5,1*d*]-1,2,3,5-tetrazin-4(3*H*)-one (3, mitozolomide, CCRG 81010, M & B 39565, NSC 353451) was the first azolotetrazinone to show good antitumour activity[12,13] in a murine screen, although results against cultures of human cancer cells have been mixed[14]. It has been the subject of Phase 1[15] and Phase 2 clinical trials and, despite displaying some activity, the further use of Mitozolomide may be limited by severe delayed toxicity[16]. It shows curative activity[17] towards the L1210 and P388 murine leukaemias and is highly potent against the TLX5 lymphoma, B16 melanoma, M5076 sarcoma, ADJ/PC6A plasmacytoma and the Colon 26 and Colon 38 tumours. Subsequent structure-activity studies[8,9] have shown a requirement for a 3-(2-chloroethyl) group for curative activity in murine tumour screeens. A 3-methyl group, as in temozolomide (4, CCRG 81045), confers less but definite activity, including the differentiation of K562 cells to a less malignant phenotype[18]. Other 3-substituents lead to the abolition of antitumour activity

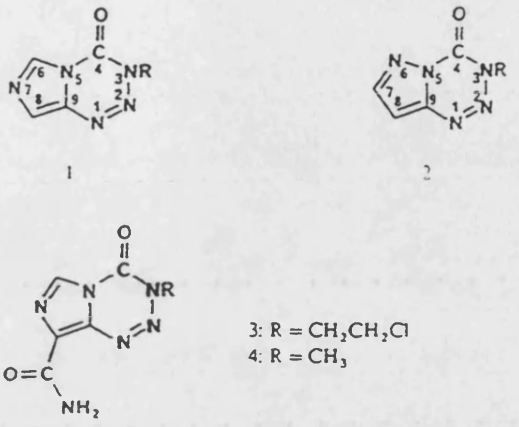
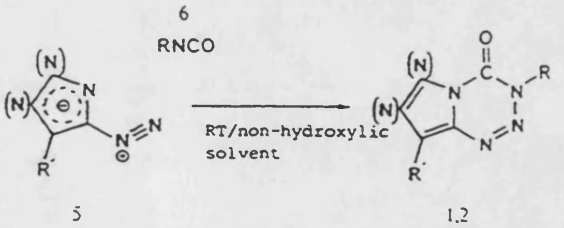


Figure 6.1 Numbering schemes of imidazotetrazinones and pyrazolotetrazinones. Structures of mitozolomide (3) and temozolomide (4).

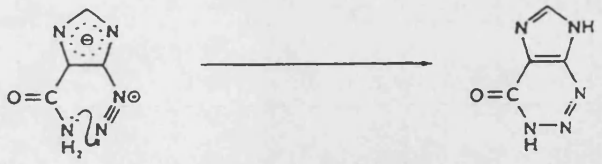
in vivo. A small alkyl group is tolerated at the 6-position, whereas there is a requirement for the 8-substituent to be electron-withdrawing (carbonyl or sulphonyl) and, preferably, to bear a hydrogen-bonding N–H moiety. In the pyrazolo series, only 3-(2-chloroethyl)pyrazolotetrazinone-8-carboxamide inhibited the growth of the P388, L1210 and M5076 tumours[10].

6.2 Synthesis

Most published syntheses of pyrazolo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-ones[5,9] and all of imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-ones[6,7,9] have involved treatment of the corresponding diazoazole (5, Scheme 6.1) with an alkyl or aryl isocyanate (6) at ambient temperature in a non-hydroxylic organic solvent, often for prolonged reaction times[9]. Systems with two liquid phases can be used for the more water-soluble diazopyrazoles and diazotriazoles[11]. Higher temperatures result in degradation of the diazoazole, particularly in the case of 5-diazoimidazole-4-carboxamide (7) which gives 2-azahypoxanthine (8) (Scheme 6.2). The cycloaddition of 5-diazoimidazole-4-carboxamide proceeds



Scheme 6.1 Synthesis of azolotetrazinones from diazoazoles and isocyanates.



7 8

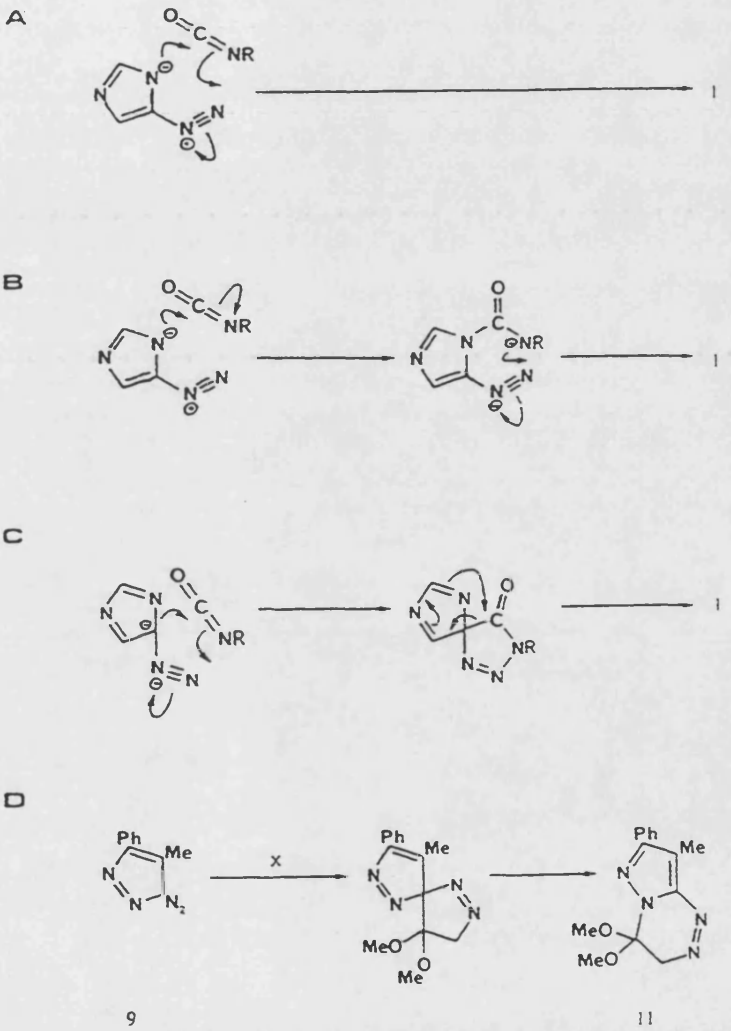
Scheme 6.2 Side reactions of 4-diazoimidazole-5-carboxamide.

smoothly in heterogeneous reactions with a wide range of isocyanates but fails with cyclohexyl isocyanate and with *tert*-butyl isocyanate[19], presumably owing to steric interactions. The corresponding bis(pyrazolotetrazinones) are formed if α,ω-diisocyanates are used[11]. Radiolabelled isotopomers of some imidazotetrazinones have been prepared with ^{14}C in the 3-substituent[20] or at C-4[21]. Synthesis of 3-unsubstituted azolotetrazinones (1, 2: R = H) by direct or indirect routes remains to be achieved. Treatment of 5-diazoimidazole-4-carboxamide with other heterocumulenes (isothiocyanates and carbodiimides) also failed[19] to furnish bicycles.

1,3-Dipolar cycloadditions have been studied extensively as routes to heterocycles[22–25]. The mechanism of the cycloaddition between diazoazoles and isocyanates to form azolotetrazinones has yet to be determined satisfactorily. Both concerted and stepwise pathways are conceivable. The fully concerted [4 + 2] route[11], shown as A in Scheme 6.3A, is unlikely owing to the considerable increase in reaction rate in the presence of hexamethylphosphoric triamide[26]. Gilchrist and Storr[27] have also generally discounted concerted mechanisms where heterocumulenes are involved as dipolarophiles. In the stepwise ionic pathway (Scheme 6.3B), there is an initial nucleophilic attack at the isocyanate carbon, followed by ring closure. More attractive is mechanism C in which an initial [3 + 2] concerted cycloaddition is followed by a [1,5] sigmatropic rearrangement; this compares closely with the mechanism[28] of the reaction between diazopyrazole (9) and 1,1-dimethoxyethene (10) to give the pyrazolo-1,2,4-triazine (11) (Scheme 6.3D).

Stepwise synthetic assembly of the tetrazinone ring in two distinct senses has been reported[11] for the pyrazolotetrazinones. In the first, involving insertion of N-2 (Scheme 6.4A), diazotisation/ring closure of carbamoylpyrazole (12) is effected by a nitrosating agent to give the pyrazolotetrazinone (13). Insertion of the carbonyl group by phosgene or its equivalent is also possible[11], converting the pyrazolyltriazene (14) to the bicycle (13) (Scheme 6.4B).

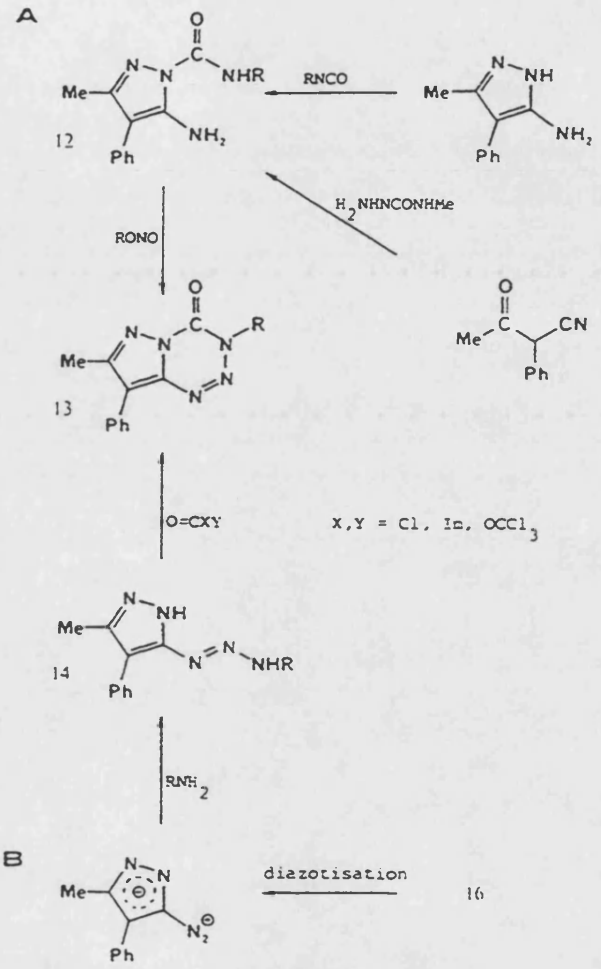
The infrared spectra[5,7,9,11] of the azolotetrazinones show that the tetrazinone carbonyl is in a relatively electron deficient environment (υ_{max} 1725–1790 cm^{-1}). The electronic absorption maximum of the 8-carbamoylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-ones occurs in the ultraviolet region between 325 nm and 338 nm and is largely independent of the nature of



9 11

Scheme 6.3 Proposed mechanisms for the reaction of diazoimidazoles with isocyanates. (A) Concerted mechanism; (B) stepwise ionic mechanism; (C) stepwise 1,3-dipolar cycloaddition followed by 1,5 shift.

the 3-substituent (aryl or alkyl). The corresponding 8-carbamoylpyrazolotetrazinones absorb[10] at shorter wavelengths (310–314 nm). In the NMR spectra of the 6-unsubstituted imidazotetrazinones, the principal characteristic signal arises from the 6-proton which resonates[7,9,19,29] between δ 8.60 and δ 9.05 ppm. The 3-CH_3 protons of temozolomide (4) give rise to a sharp singlet at δ 3.90; this strong deshielding again reflects the electron-deficient and electrophilic nature of the 1,2,3,5-tetrazin-4-one ring. In the mass spectra,



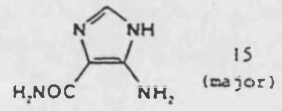
Scheme 6.4 Alternative synthetic approaches to pyrazolotetrazinones.

the factor determining the fragmentation pathway is the nature of the 3-substituent. 3-Arylimidazotetrazinones do not give a molecular ion[19] but only ions corresponding to the products of retrocyclisation. In contrast, the 3-alkyl imidazotetrazinones and pyrazolotetrazinones show abundant molecular ions[10,19]. Interestingly, a major ion (often the base peak) in the mass spectra of 3-(2-chloroethyl)azolotetrazinones is the 3-methyleneiminium ion[10,21,30]. X-ray crystal structure determinations have been reported for mitozolomide (3)[30], for the analogous 3-(2-methoxyethyl) compound[31] and for temozolomide (4)[31]. The two rings of each compound lie effectively in one plane which also contains the atoms of the carboxamide moiety.

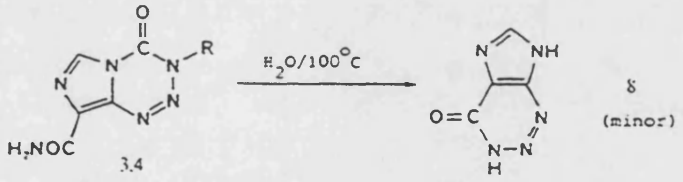
6.3 Reactions involving opening of the tetrazinone ring

Comprehensive studies have been carried out on the reactivity of the imidazotetrazinones. However, study of the chemistry of the pyrazolotetrazinones has been limited. Pyrazolotetrazinones are generally[10,19] stable to light; whereas the imidazo compounds are moderately photolabile[19], giving rise to red pigments, presumably azoimidazoles. Thermolysis of azolotetrazinones leads to retrocyclisation[7].

Treatment of azolotetrazinones with nucleophiles results in opening of the tetrazinone ring, whereas acidic or electrophilic reagents usually permit this structure to be retained. Stone[19] determined the half-lives of 8-carbamoylimidazotetrazinones in aqueous phosphate buffer (pH 7.4) in the dark and found them to be in the range 2.9 min to 155 min. These data compare well with the rates of hydrolysis of mitozolomide in plasma[32] and with the observed great sensitivity to pH of the rate of hydrolysis of mitozolomide[33] ($t_{1/2}$ = 2.1 h at pH 7.0 and 0.9 h at pH 7.5). Baig and Stevens[34] reported that mitozolomide (3) and temozolomide (4) both decomposed rapidly in boiling water to give 5-aminoimidazole-4-carboxamide (15, Scheme 6.5) as the major product, together with azahypoxanthine (8) and the imidazolylazoimidazole (16). The minor products arise from initial thermal 6π electrocyclic ring opening giving 5-diazoimidazole-4-carboxamide. The tentative identification of the inter-



15
(major)



3,4

8
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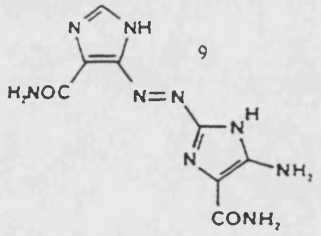

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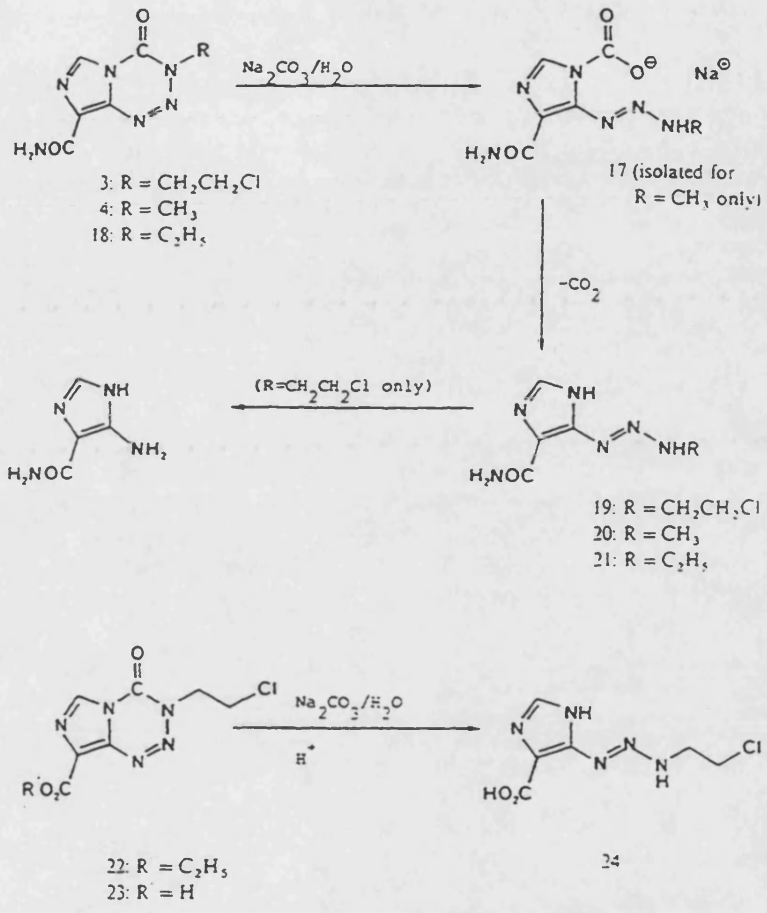
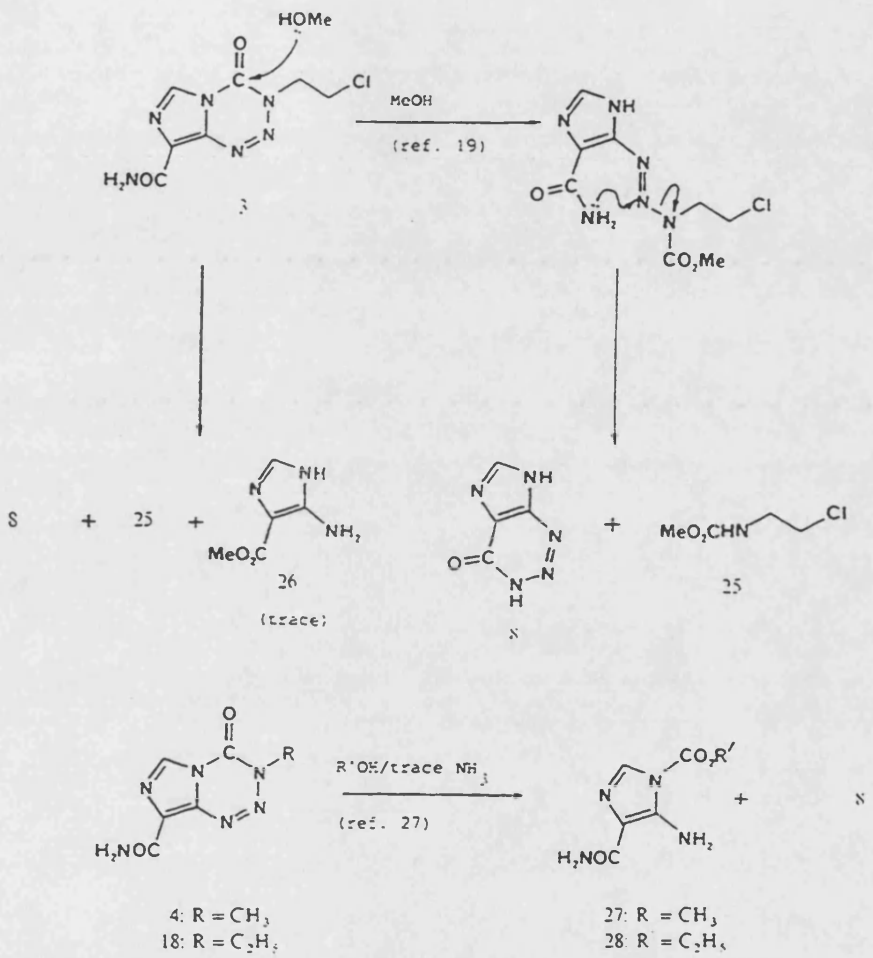
Scheme 6.5 Decomposition of mitozolomide (3) and temozolomide (4) in water.



Scheme 6.6 Hydrolysis of imidazotetrazinones in aqueous base.

mediate carbamate salt (**17**) in the hydrolysis of temozolomide in cold aqueous sodium carbonate shows that the formation of triazene (**20**) involves initial nucleophilic attack of water (or hydroxide) at the carbonyl group at the 4-position of the tetrazinone ring (Scheme 6.5). The main products of such base-catalysed hydrolysis[34] of the 3-alkyl-8-carbamoylimidazotetrazinones (**3**, **4**, **18**) were the corresponding triazenes (**19**–**21**). The ester (**22**) and the carboxylic acid (**23**) both gave the triazene carboxylic acid (**24**). Base-catalysed hydrolysis of pyrazolotetrazinones proceeds similarly[11]. The 2-chloroethyltriazene (MCTIC, **19**) arising from hydrolysis of mitozolomide is itself labile[33] to hydrolysis at pH 7.4 giving a mixture of 2-chloroethanol and (**15**) with the evolution of nitrogen[35].

The imidazotetrazinones react in a similar manner with alcohols, although the reported rates of reaction vary from worker to worker (Scheme 6.7). The

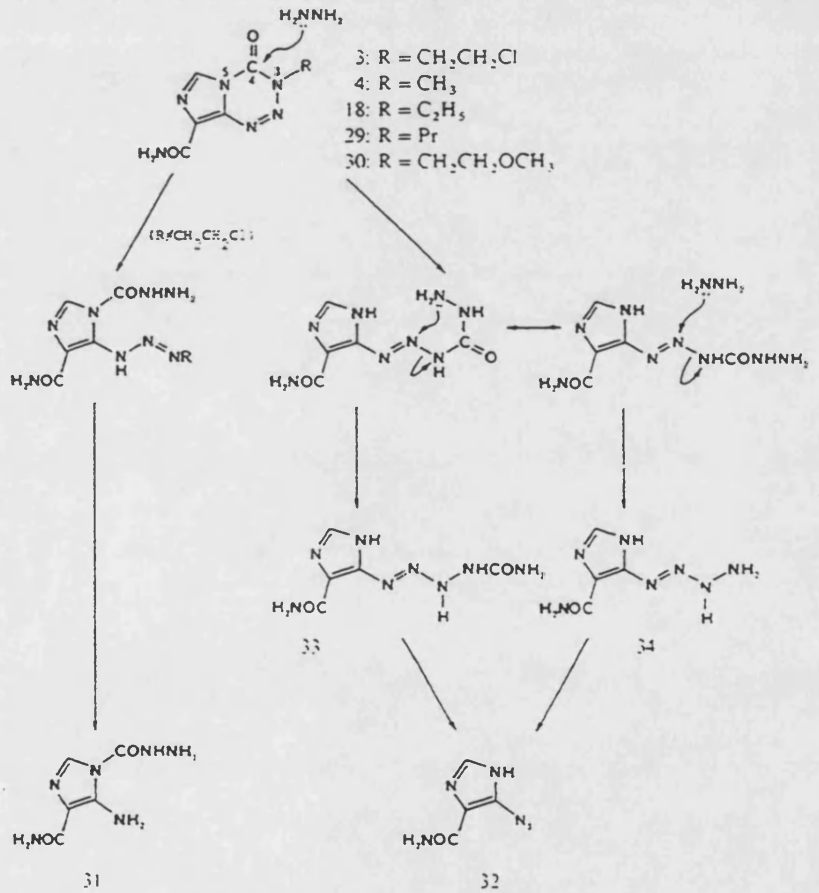


Scheme 6.7 Alcoholysis of imidazotetrazinones.

products from the treatment of mitozolomide with cold methanol were reported by Stone[19] to be 2-azahypoxanthine (8) and methyl *N*-(2-chloroethyl) carbamate (25); in boiling methanol, a small trace of methyl 5-aminoimidazole-4-carboxylate (26) was observed[7]. However, Baig[34] reported the 3-methyl and 3-ethyl imidazotetrazinones (4, 18) to be very stable to boiling methanol. The rate of reaction with alcohols is greatly enhanced by addition of a trace of ammonia[19,29]. Under these base-catalysed conditions, the main product from the 3-methyl and 3-ethyl imidazotetrazinones was still 2-azahypoxanthine but the alkoxycarbonylimidazoles (27, 28) were also formed[34]. The characterisation of these products of alcoholysis was complicated owing to conflicting identifications[36,37] of the products of alkoxy-

carbonylation of 5-aminoimidazole-4-carboxamide (15). Mitozolomide gave exclusively 2-azahypoxanthine, the product of cleavage of the 4,5 bond of the tetrahedral intermediate. It is interesting to note the lengths[30,31] of the 3, 4 (1.374 ± 0.003 Å) and 4,5 (1.392 ± 0.003 Å) bonds of the imidazotetrazinones in this context as indicators of bond order and bond energy and that corresponding lengths are not appreciably different between analogues.

The reactivity of the 3-(halo)alkylimidazotetrazinones with a representative nitrogen nucleophile, hydrazine[34], parallels that with the oxygen nucleophiles detailed above. The four 3-alkyl compounds (4,18,29,30) react slowly to form the carbohydrazide (31) through cleavage of the 3,4 bond (Scheme 6.8). In contrast, hydrazine caused fission of the 4,5 bond of mitozolomide (3), the final isolated product being 4-azidoimidazole-5-carboxamide (32), presumably via one of the tetrazenes (33, 34). This mechanistic proposal is supported by the



Scheme 6.8 Hydrazinolysis of imidazotetrazinones.

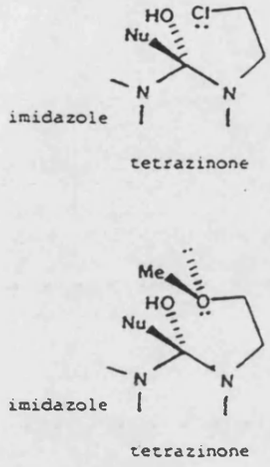
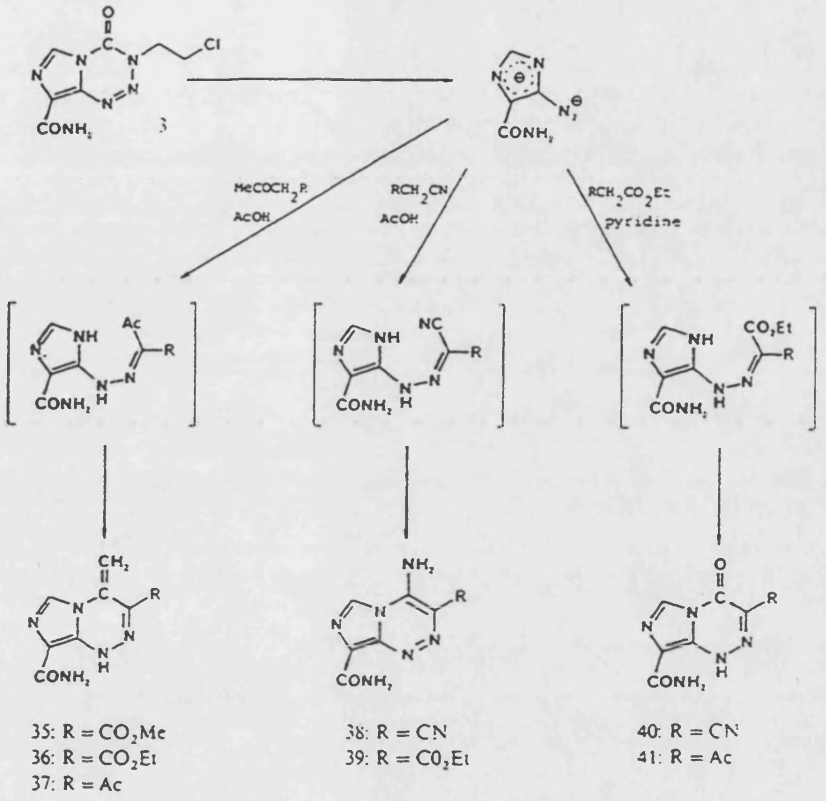


Figure 6.2 Stereoelectronic requirements for intramolecular nucleophilic catalysis in the reaction of 3-(2-chloroethyl) imidazotetrazinones with oxygen and nitrogen nucleophiles. Steric interaction between the methyl group (Me) and the nucleophiles (Nu) preclude this anchimeric assistance in 3-(2-methoxyethyl) analogues.

ready synthesis of this azide from 5-diazoimidazole-4-carboxamide (7) and hydrazine[38]. An explanation for the apparently greater lability of the 4,5 bond in mitozolomide, when compared with the 4,5 bond in the 3-alkylimidazotrazinones, during reaction with alcohols and hydrazine may be that the chlorine atom in the tetrahedral intermediate can, owing to geometric constraints, only provide an intramolecular nucleophilic catalysis (anchimeric assistance) for cleavage of this bond (Figure 6.2). Clearly, this bond-weakening effect is not possible when the 3-substituent does not bear a heteroatom. The ethereal oxygen atom in the 2-methoxyethyl sidechain may be unable to perform this assistance owing to steric interactions from the methyl group in the stereoelectronically required reacting conformation. In the pyrazolotetrazinone series, however, the 4,5 bond is intrinsically weaker than the 3,4 bond[11].

Sandmeyer-type products (4-haloimidazole-5-carboxamides) are obtained when mitozolomide is treated with bromide or iodide ions in hot acetic acid[34]. It has been suggested this represents reaction of the thermal cycloreversion product, 5-diazoimidazole-4-carboxamide, rather than direct reaction of the tetrazinone although this is not completely clear. Products arising from the formal trapping of 5-diazoimidazole-4-carboxamide, generated from mitozolomide, by carbon nucleophiles have also been observed[34] during reactions in hot acetic acid and in boiling pyridine (Scheme 6.9). The regioselectivity of the subsequent cyclisations to form imidazotriazines (35–41) is determined by the acidic or basic catalysis provided by the solvents and by the nature of the substituents attached to the active methylene function.

The effect of powerful oxidants on azolotetrazinone rings has not been



Scheme 6.9 Reactions of mitozolomide (3) with carbon nucleophiles.

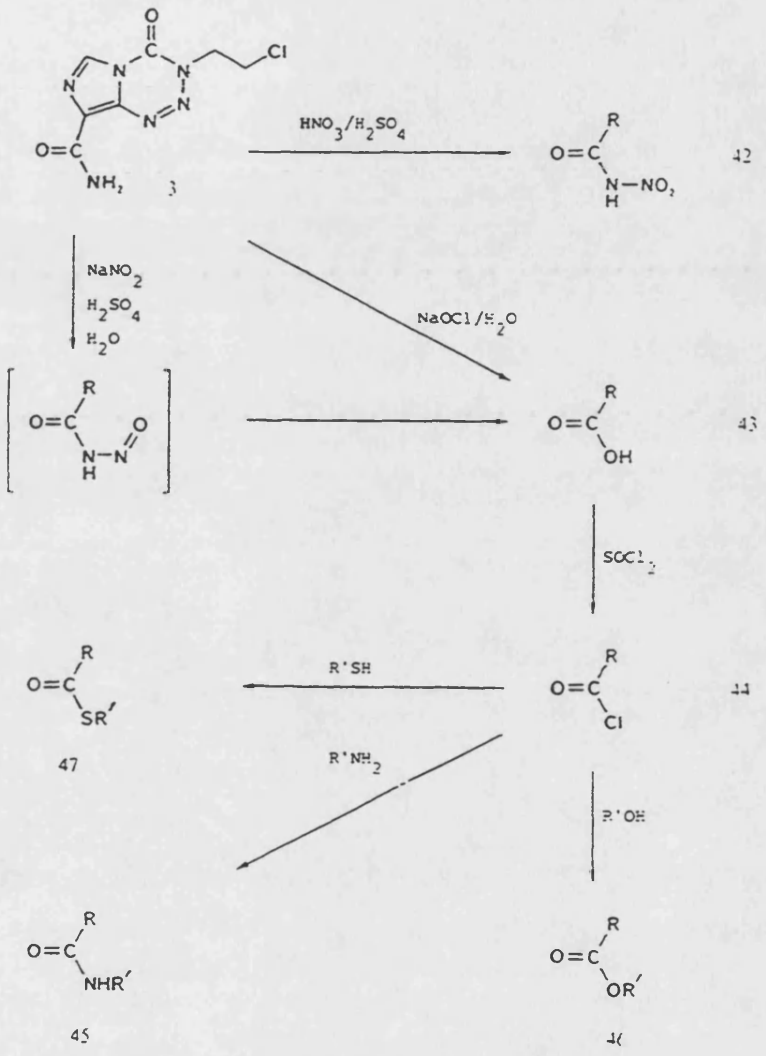
reported. Similarly, the effect of reducing agents is unknown but simple triazenes are unaffected by complex hydrides[39].

In summary, the chemistry of the imidazotetrazinones with nucleophiles is characterised by opening of the tetrazinone ring. The immediate products are frequently subject to further transformation under the reaction conditions.

6.4 Reactions involving retention of the tetrazinone ring

Acidic or electrophilic reagents usually permit the imidazotetrazinone ring structure to be retained. Indeed, mitozolomide (3) has been recovered unchanged from warm concentrated sulphuric acid, in striking contrast to the considerable acid-lability of the open-chain triazenes[40]. Presumably, this reflects the basicity of the N = N–N group in the different systems.

Some useful chemical transformations of functional groups attached to azolotetrazinones have, however, been accomplished using acidic and electrophilic reagents[29]. Treatment of mitozolomide with concentrated 'nit-



Scheme 6.10 Reactions of mitozolomide (3) with electrophiles. R = 3-(2-chloroethyl)-4-oxoimidazo[5,1-*d*]-1,2,3,5-tetrazin-8-yl.

rating mixture' gave only the *N*-nitroamide (42) (Scheme 6.10). This compound was found to be stable to mild hydrolytic conditions and to attempted rearrangement[32]. Nitrosation at carboxamide nitrogen affords a convenient synthesis of the carboxylic acid (43); this hydrolysis can also be effected by aqueous sodium hypochlorite. The chemistry of this carboxylic acid is straightforward, provided that aqueous basic conditions are avoided. The acid chloride (44), formed by treatment with thionyl chloride, reacts vigorously[42]

with the appropriate nucleophiles to give amides, esters and thioesters (45–47).

Metabolic oxidation of the *N*-methyl groups of the *N,N*-dimethylcarboxamide analogue of mitozolomide by cytochrome P_{450} requiring enzymes of rodent systems has been described recently by Horspool[40]. The characterised products are the *N*-methylamide and mitozolomide presumably arising via *N*-(hydroxymethyl) intermediates in analogy with other aromatic amides[41].

Efficient deprotection of *N*-(4-methoxybenzyl) protected imidazotetrazinone-8-carboxamides and -8-sulphonamides by a standard[43] method of acid-catalysed nucleophilic substitution has been reported[29]. When the protecting group is attached directly to the tetrazinone ring, as in 3-(4-methoxybenzyl)imidazotetrazinones, this method appears to fail and the 3-unsubstituted imidazotetrazinone is not formed[29].

In summary, the chemistry of the azolotetrazinones with electrophiles and with acids is confined to interactions with peripheral functional groups.

6.5 Relationship of the chemistry of the azolotetrazinones to their biochemistry

The potent cytotoxicity of the lead compound, mitozolomide (3), has been attributed to interstrand cross-linking of DNA[44,45]. Tisdale[46] has recently extended this study to a wider range of cell lines and to the active antitumour agent temozolomide (4), and to the 3-ethyl analogue (18) which is cytotoxic *in vitro* but is not an antitumour agent *in vivo*. Discussions of the relationship of the chemistry to the (bio)chemical modes of action of the imidazotetrazinones must therefore take account of these observations and of the structure/antitumour activity relationship.

Of the two types of electrophilic biochemical reactivity (carbamoylation and alkylation) considered by Horgan and Tisdale[47], the former was discounted on the grounds that mitozolomide does not inhibit glutathione reductase, chymotrypsin and γ-glutamyltranspeptidase under conditions where these enzymes are inhibited by carbamoylating agents. The partial inhibition of an esterase of EMT6 cells by mitozolomide may, however, indicate some carbamoylation by the reagent[48]. The most likely reactive antitumour metabolite or metabonate of mitozolomide (3) is the *N*-(2-chloroethyl)triazene (MCTIC, 29), the known product of aqueous hydrolysis. The cytotoxic activity of mitozolomide has then been explained[47] in terms of the reaction of this potent alkylating electrophile with nucleophilic sites on DNA, in particular with O^6 of guanine bases, with subsequent cross-linking of the two strands of the macromolecule. The differential sensitivity of the cell lines VA13 (sensitive, Mer^-) and IMR90 (more resistant, Mer^+) to mitozolomide is then consistent with the ability of the latter to repair the O^6-(2-chloroethyl) lesion prior to the cross-linking reaction. Temozolomide (4) is thought to exert its cytotoxicity through a similar mechanism involving the

monomethyltriazene (20); although cross-linking is precluded. The 3-ethyl analogue (18), is an alkylating agent towards RNA, DNA and proteins and is a cytotoxin *in vitro*; however, alkylation of the O^6 position of guanine is not involved[20,46].

True enzymic metabolism of the imidazotetrazinones, as distinct from chemical change in physiological media, has been reported to result either in activation of the substrate as an antitumour agent or in deactivation, depending on the precise nature of the peripheral functional groups[49–51].

6.6 Concluding remarks

The azolotetrazinones represent a highly reactive series of heterocycles, both chemically and biologically. Suitably substituted, they show both electrophilic and nucleophilic character, with opening or retention of the ring system, respectively. Both the antitumour efficacy of some members of the class and their toxicity towards cells and organisms result from potently alkylating electrophiles generated by hydrolytic chemical reactions *in vivo*. Further detailed analysis of the interactions of azolotetrazinones and their metabonates with DNA may be necessary to explain satisfactorily the observed structure-activity relationship at a molecular level and to permit the design of a more selective and, therefore, more therapeutically useful agent.

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Syn Selectivity in the Epoxidation of 1-(Cyclohex-2-enyl)-2-nitroimidazole Mediated by Hydrogen-Bonding

M. D. Threadgill and P. Webb

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syn-Selectivity in the Epoxidation of 1-(Cyclohex-2-enyl)-2-nitroimidazole mediated by Hydrogen Bonding

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Epoxidation of 1-(cyclohex-2-enyl)-2-nitroimidazole with 3-chloroperoxybenzoic acid occurs exclusively *syn* to the nitroimidazole in the absence of water; amines react only with the *syn* epoxide.

The stereochemistry of epoxidation by peroxy acids of many cyclohexenes bearing bulky 3-substituents is generally governed by steric factors, the geometrical isomer with the epoxide oxygen *anti* to the 3-substituent being the major or exclusive product when the substituent is non-polar or a hydrogen-bond acceptor.[1–4] Hydrogen bonding is, however, involved in the exclusive *syn*-epoxidation of cyclohexen-3-ol;[4–6] similar control may be taking place in the *syn*-aziridination of this substrate.[7] One exception to this rule is the dependence on solvent of the epoxidation of 3-(*tert*-butyldimethylsilyloxy)cyclohexene by peroxytrifluoroacetic acid.[8] As part of a continuing programme of design, synthesis and evaluation of new radiosensitisers and bioreductively activated cytotoxins based on nitro-heterocycles and, in particular, study of conformationally controlled analogues of the lead compound RSU 1069 [α-(aziridin-l-ylmethyl)-2-nitroimidazole-1-ethanol],[9] we required the diastereoisomers of 1-(3-aziridinyl-2-hydroxycyclohexyl)-2-nitroimidazole and expected that those with aziridine *trans* to the hydroxy group could be formed from the appropriate epoxide.

1-(Cyclohex-2-enyl)-2-nitroimidazole **2**† was prepared by alkylation of the potassium salt of 2-nitroimidazole **1** under phase-transfer conditions,‡ in a modification of the general method of Parrick *et al.*[10] (Scheme 1). Treatment of cycloalkene **2** with the commercial grade of 3-chloroperoxybenzoic acid (MCPBA) (containing *ca.* 35% water) in boiling dichloromethane gave§ a mixture of epoxides **3a** and **3b** in ratios between 2 : 1 and 3 : 1. When the epoxidation was carried out using dried samples of the peroxyacid, the sole epoxide formed was **3a**. Mixtures of **3a** and **3b** could be separated analytically but not preparatively by chromatography. Assignment of the diastereoisomeric identities of the major and minor components of the product mixture was made by nuclear Overhauser enhancement (NOE) measurements in the 270 MHz ^{1}H NMR spectra (Fig. 1), which confirmed the assignments of the resonances due to the cyclohexane 1-H, 2-H and 3-H. Particularly diagnostic are the values of the NOEs between the 1-H and 2-H in **3a,b**; the value for the *syn*-isomer **3a** is nearly three-fold larger than the corresponding value for the *anti*-isomer **3b**. These data are consistent with the distance between 1-H and 2-H in the *cis*-arrangement in **3a** being much shorter than the 1-H to 2-H (*trans*) distance in **3b** in the geometry required by the pseudochair conformation. Treatment of mixtures of **3a** and **3b** with aziridine (**CAUTION**) or with the less sterically demanding nucleophile methylamine in boiling ethanol resulted in the consumption exclusively of

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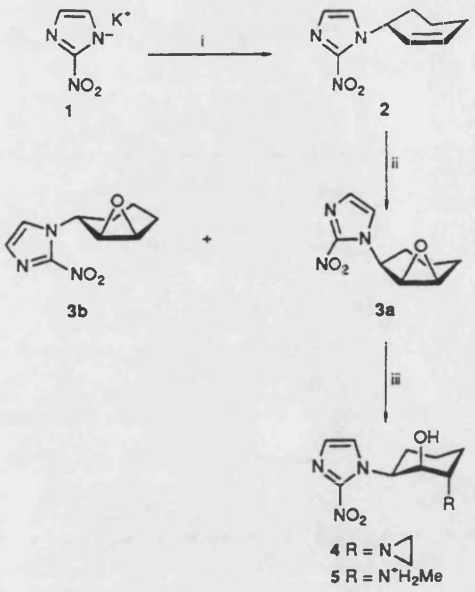
† New compounds were chromatographically homogeneous and gave satisfactory ^{1}H NMR and accurate mass spectra. All compounds were racemic.

‡ Salt **1** was stirred with 18-crown-6 and 3-bromocyclohexene in acetonitrile for 24 h, giving, after work-up, **2** (31%) as a gum; ^{1}H NMR ($CDCl_3$) δ 1.5–2.4 (6 H, m, 4,5,6-H), 5.70 (1 H, m) and 5.80 (1 H, m) (1,2-H), 6.20 (1 H, ddd, *J* 8, 4, and 3 Hz, 3-H), 7.10 (1 H, s, imidazole 4-H) and 7.25 (1 H, s, imidazole 5-H).

§ Typical experiment. Compound **2** was boiled under reflux in dichloromethane with MCPBA [commercial sample: MCPBA (55% w/w), 3-chlorobenzoic acid (10%) and water (35%)] (3 equiv.) for 20 h to give, after aqueous work-up, a mixture of **3a** and **3b** (48%) in the ratio *ca.* 3 : 1. **3a**: ^{1}H NMR ($CDCl_3$) δ 1.5–2.2 (6 H, m, 4,5,6-H), 3.43 (2 H, br s, 2,3-H), 5.56 (1 H, m, 1-H), 7.17 (1 H, s, imidazole 4-H), and 7.57 (1 H, s, imidazole 5-H). **3b**: ^{1}H NMR ($CDCl_3$) δ 1.45–1.65 (3 H, m, 5*ax*,5*eq*,6*ax*-H), 1.95 (1 H, ddt, 4*ax*-H), 2.12 (1 H, m, 6*eq*-H), 2.22 (1 H, ddt, 4*eq*-H), 3.25 (1 H, d, 2-H), 3.43 (1 H, m, 3-H), 5.27 (1 H, dd, 1-H), 7.22 (1 H, d, imidazole 4-H) and 7.24 (1 H, br s, imidazole 5-H).



Scheme 1 Synthesis and reactions of epoxide stereoisomers **3a,b**. Epoxide **3b** is only formed in the presence of water. *Reagents*: i, 3-bromocyclohexene–18-crown-6–acetonitrile; ii, 3-chloroperoxybenzoic acid–dichloromethane; iii, RH–ethanol–triethylamine.

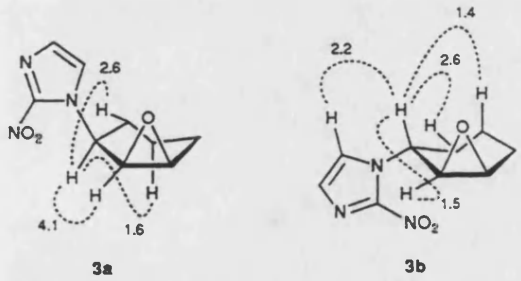


Fig. 1 NOEs for epoxides **3a,b**. The values of the NOEs are shown as percentages on the dashed lines joining pairs of hydrogens.

the major isomeric epoxide, giving **4**† and **5**† in 81 and 52% yields respectively (based on the content of *syn*-isomer **3a** in the starting mixture of **3a** and **3b**). ^{1}H NMR spectra¶ demonstrated that the addition products **4** and **5** have the structures and conformations shown in Scheme 1. In particular, the 2-D carbon–proton chemical shift correlation study on **5** showed *inter alia* that the proton resonances at δ 3.26. 4.19 and 5.33 are due to C–H, whereas that at δ 5.89 is not and can be assigned to O–H. Irradiation of the signal at δ 4.19 causes collapse of couplings at δ 5.89 and 5.33; thus, these protons comprise a CH–CH–OH system. The resonance at δ 5.33 is assigned to 1-H by chemical shift analogy with open-chain analogues (*e.g.* RSU 1069) and by observation of a *trans* diaxial coupling to 6*ax*-H. Therefore, the OH group is at

¶ **4**: ^{1}H NMR [$(CD_3)_2SO$] δ 1.12 (1 H, dd, aziridine-H *syn* to N–cyclohexane bond), 1.16 (1 H, dd, aziridine-H *syn* to N–cyclohexane bond), 1.45–1.90 (7 H, m, 2 × aziridine-H *anti* to N–cyclohexane bond + 4*ax*,4*eq*,5*ax*,5*eq*,6*eq*-H), 2.17 (1 H, dq, 6*ax*-H), 3.34 (1 H, m, 3-H), 3.91 (1 H, m, 2-H), 5.11 (1 H, d, OH), 5.49 (1 H, d *ca.* t, 1-H), 7.15 (1 H, d, imidazole 4-H), and 7.67 (1 H, d, imidazole 5-H). **5**: ^{1}H NMR [$(CD_3)_2SO$] δ 1.6–1.9 (5 H, m, 4*ax*,4*eq*,5*ax*,5*eq*,6*eq*-H), 2.19 (1 H, br q, 6*ax*-H), 2.63 (1 H, br s, NCH_3), 3.26 (1 H, m, 3-H), 4.19 (1 H, m, 2-H), 5.33 (1 H, 1-H), 5.89 (1 H, d, OH), 7.20 (1 H, s, imidazole 4-H), 7.72 (1 H, s, imidazole 5-H), and 9.20 (1 H, br) and 9.35 (1 H, br) ($^+NH_2$).

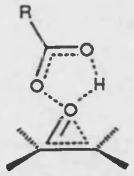


Fig. 2 General transition state for epoxidation of alkenes by peroxy acids

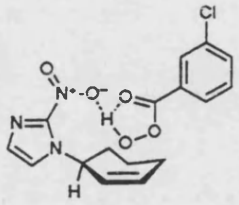


Fig. 3 Stereocontrol by hydrogen bonding in the reacting complex between cycloalkene **2** and MCPBA

C-2 and 5 has the structure shown; other regio- and stereo-isomers are not consistent with these observations. Both compounds have approximately the conformations indicated in Scheme 1, with nitroimidazole in the equatorial arrangement and hydroxy and nitrogen functions axial; this contrasts with the conformation reported by Lier *et al.*[1] for the analogous *t*-3-amino-*c*-2-phenoxycyclohexan-*r*-1-ol.

The mechanism of the epoxidation of alkenes is considered to involve the peroxy acid in the conformation with an intramolecular hydrogen bond, with rates of reaction being lower in hydrogen-bond accepting solvents.[11] There is also general consensus on the structure of the transition state being similar to that shown in Fig. 2, although 1,3-dipolar cycloaddition has also been proposed.[12] The specificity of epoxidation of **2** by MCPBA in the anhydrous reaction can be rationalised in terms of control through the hydrogen-bonded complex shown in Fig. 3. Examination of models of this structure reveals that the peroxide oxygen is appositely located for the epoxidation process shown in Fig. 2. The presence of trace amounts of water in the reaction mixture, occasioned by the use of undried commercial MCPBA, would tend to disrupt this controlling hydrogen bond, relatively disfavouring epoxidation *syn* to the nitroimidazole.

Thus, stereocontrol is possible through hydrogen bonding of the weak acid MCPBA to an appropriately located hydrogen-bond acceptor, such as the nitro group. This contrasts with the reported[8] lack of such control with MCPBA but good control with the stronger acid peroxytrifluoroacetic acid in the epoxidation of 3-(*tert*-butyldimethylsilyloxy)cyclohexene; this selectivity is abolished when a hydrogen-bond acceptor, such as tetrahydrofuran, is used as the solvent. The demonstration of *syn*-stereospecificity in the epoxidation of **2** with MCPBA suggests either that the nitro group is a more powerful hydrogen-bond acceptor than is a silyl alkyl ether or that the geometry of the intermediate complex is more appropriate for reaction. Further studies on the generality and mechanism of this stereospecificity are being pursued.

The complete lack of reactivity of the amine nucleophiles with isomer **3b**, compared with ready addition to isomer **3a**, is consistent with a preference for ring-opening to give a *trans*-diaxial product in analogy with precedents for opening of other epoxides[3,4,13] with nucleophiles. The corresponding *trans*-diaxial reaction of nucleophiles with the *anti*-isomer **3b**

would require approach to C-2: this is precluded on steric grounds.

The epoxidation of cyclohexene **2** with MCPBA in the presence or absence of small amounts of water represents a new opportunity for exploitation of hydrogen bonding of this readily handled peroxy acid in stereocontrolled synthesis.

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PUBLICATION 45

Herstellung des Arzneimittels Hexamethylmelamin mit deuteriertem Seitenstück

M. D. Threadgill

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Isotopenpraxis 27 (1991) 3, pp. 143 – 144

Herstellung des Arzneimittels Hexamethylmelamin mit deuteriertem Seitenstück

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Bei der Umsetzung von N,N,N',N',N''-Pentamethyl-1,3,5-triazin-2,4,6-triamin mit Butyllithium und Trideuteroiodmethan entsteht in großer Ausbeute N,N,N',N',N''-Pentamethyl-N''-(trideuteromethyl)-1,3,5-triazin-2,4,6-triamin, das Arzneimittel Hexamethylmelamin mit deuteriertem Seitenstück.

Treatment of N,N,N',N',N''-Pentamethyl-1,3,5-triazin-2,4,6-triamine with butyllithium and trideuteroiodomethane gives N,N,N',N',N''-Pentamethyl-N''-(trideuteromethyl)-1,3,5-triazin-2,4,6-triamine, a deuterated analogue of the anticancer drug hexamethylmelamine, in high yield.

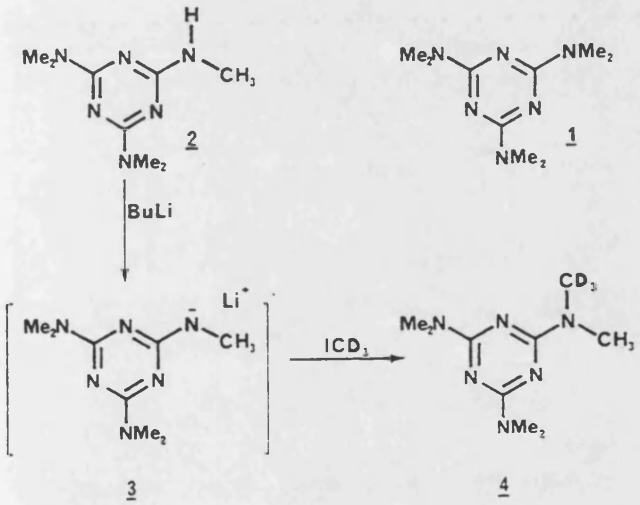
Keywords

deuterium; drugs; labelled compounds; labelling; lithium compounds; melamine; metabolism; neoplasms; synthesis

*) Sonderdruckanforderungen und Korrespondenz an Dr. M. D. Threadgill, School of Pharmacy, University of Bath, Claverton Down, Bath BA2 7AY, Großbritannien

Hexamethylmelamin (HMM; **1**) ist ein Arzneimittel mit Wirksamkeit gegen verschiedene maligne Erkrankungen besonders Eierstockcarzinom [1]. Die metabolische Hydroxylierung von N-CH_3 Gruppen ist unentbehrlich für diese Aktivität [2]. Um Versuche über den Mechanismus der metabolischen Oxidation von Hexamethylmelamin in der Säugetierleber durchzuführen und die metabolische Veränderung an den Kohlenstoffatomen nächst Stickstoffatomen zu studieren [3–6], waren deuterierte Derivate erforderlich. Solche Derivate von Verbindung **1** sind noch nicht beschrieben worden, obwohl die Herstellung von beiden möglichen [^{14}C]-markierten Derivaten nach der klassischen Methode die auf der Reaktion von 2,4,6-Trichloro-1,3,5-triazin mit Dimethylamin beruht, in mittlerer Ausbeute in der Literatur beschrieben ist [7, 8].

In der vorliegenden Arbeit wurde die isotopisch markierte Gruppe in der letzten Herstellungsstufe mit dem Restmolekül verbunden. In zwei vorherbeschriebenen Stufen wurde Pentamethylmelamin (**2**) aus 2,4,6-Trichloro-1,3,5-triazin hergestellt, (i) durch Umsetzung mit Dimethylamin bei Raumtemperatur; (ii) durch Reaktion des Zwischenprodukts *N,N,N',N'*-Tetramethyl-1,3,5-triazin-2,4,6-triamin mit Methylamin unter Erhitzen. Umsetzung der Verbindung **2** mit Butyllithium entwickelt das Anion **3**. Dieses Anion reagiert schnell und ruhig mit Trideuterojodomethan bei Raumtemperatur (siehe Schema) und die Reinigung des *N,N,N',N',N''*-pentamethyl-



N''-(trideuteromethyl)-1,3,5-triazin-2,4,6-triamins (**4**) war einfach. Diese Beobachtung widerspricht dem Vorschlag von *Honda* et al. [9], demzufolge Natriumamid und Dimethylsulphat als hartes Electrophil für Methylierung des 1,3,5-triazin-2,4,6-triamins in großen Ausbeuten erforderlich sind.

Experimenteller Teil

Herstellung von *N,N,N',N',N''*-Pentamethyl-*N''*-(trideuteromethyl)-1,3,5-triazin-2,4,6-triamin (**4**). – Zu einer Lösung von *N,N,N',N',N''*-Pentamethyl-1,3,5-triazin-2,4,6-triamin [10] (570 mg, 2.91 mmol) in wasserfreiem Tetrahydrofuran (aus Calciumhydrid frisch destilliert; 10 mL) wurde Butyllithium (2.0 mL; 1.5 M in Hexan; 3.0 mmol) bei Raumtemperatur zugetropft. Nach 30 Min wurde Trideuterojodomethan (99 atom%; Aldrich Chemical Co.; 500 mg, 3.45 mmol) zugesetzt. Das Reaktionsgemisch wurde bei Raumtemperatur für 18 Std. gerührt. Das Lösungsmittel wurde unter vermindertem Druck abgedampft. Der Rückstand wurde in Dichloromethan aufgelöst, mit Wasser geschüttelt und mit wasserfreiem Na_2SO_4 getrocknet. Der Abdampfrückstand wurde säulenchromatographisch (SiO_2; Hexan : Diethylester; 1 : 1) vereinigt: *N,N,N',N',N''*-pentamethyl-*N''*-(trideuteromethyl)-1,3,5-triazin-2,4,6-triamin (**4**) (470 mg, 75%); weiße Kristalle; Schmp. 166 °C (lit. [11] 171 – 172 °C für die unmarkierte Substanz); IR (Nujol) ν 2800 (NCH_3), 2045 (NCD_3) cm^{-1}; ^{1}H NMR ($CDCl_3$) 3.10 (s, 5 × CH_3); MS (EI) *m/z* 213 (100%) (M^+). Diese Substanz war dünnschichtchromatographisch identisch mit der unmarkierten Substanz (**1**), die durch Reaktion von 2,4,6-Trichloro-1,3,5-triazin mit Dimethylamin hergestellt wurde.

Anerkennung

Der Autor dankt Herrn Professor *Andreas Gescher* für hilfreiche Diskussion und Herrn *Adrian P. Gledhill* für ausgezeichnete technisch Hilfe.

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PUBLICATION 46

Aziridinyl Nitropyrroles and Nitropyrazoles as Hypoxia-Selective Cytotoxins and Radiosensitizers

M. A. Naylor, M. A. Stephens, I. J. Stratford, A. Keohane, P. O'Neill, M. D. Threadgill, P. Webb, E. M. Fielden and G. E. Adams

Anti-Cancer Drug Design, **1991**, *6*, 151-167.

Anti-Cancer Drug Design (1991), 6, 151–167

Aziridinyl nitropyrroles and nitropyrazoles as hypoxia-selective cytotoxins and radiosensitizers

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Summary: A series of 1- and 2-substituted 4- and 5-nitropyrroles and 3- and 4-nitropyrazoles has been prepared and evaluated *in vitro* as radiosensitizers of hypoxic cells and as bioreductively-activated cytotoxins. Both the nitropyrroles and the nitropyrazoles were considerably less effective, based upon the differential between hypoxic and aerobic toxicity, than were similar 2-nitroimidazoles bearing alkylating moieties. The trends in radiosensitizing efficiency observed for both classes of drugs corresponded with their one-electron reduction potentials (E^1_7) as measured by pulse radiolysis, although they were generally more effective than predicted from previous correlations of E^1_7 with sensitizing efficacy and reactivities. Furthermore, the enhancement of sensitizing efficiency by the incorporation of alkylating groups is considerably greater than has been observed for nitroimidazoles. α-[(1-Aziridinyl)methyl]-3-nitropyrazole-1-ethanol (**10**, $E^1_7 = -456$ mV) and methyl 5-nitro-1-(cyclopropylcarbonyl)pyrrole-2-carboxylate (**25**, $E^1_7 = -326$ mV) were the most effective radiosensitizers *in vitro*. Only 3-((*cis*-2,3-dimethyl-1-aziridinyl) methyl)-1-oxo-3,4-dihydro-6-nitro-1-H-pyrrolo [2,1-c] oxazine (**22**) and methyl 5-nitro-1-(cyclopropylcarbonyl)pyrrole-2-carboxylate (**25**) showed significant bioreductively-activated cytotoxicity, with differentials of 3.5. Although these differential toxicities were coupled with significantly lower aerobic toxicity compared with similar 2-nitroimidazoles, this series was not deemed effective enough to warrant further evaluation. The electron affinity and radiosensitization could be manipulated by chemical design but hypoxia-selectivity was not clearly related to these properties.

The development of nitroheterocyclic drugs in the field of radiosensitization and bioreductively-activated cytotoxic drugs in cancer chemotherapy is dominated by the nitroimidazoles (Adams *et al.*, 1984; Coleman *et al.*, 1984; Ahmed *et al.*, 1986; Chaplin *et al.*, 1986; Newman *et al.*, 1986; Roberts *et al.*, 1986; Jenkins, 1989; Jenkins *et al.*, 1990). These compounds act as sensitizers of normally radiation-resistant hypoxic cells with little or no effect on normal well oxygenated cells. The ability of the drugs to act in this way is known to be related to their reduction potentials (Adams *et al.*, 1976, 1979). 2-Nitroimidazoles have the further advantage of selective cytotoxicity, by being anaerobically reduced to toxic metabolites in hypoxic tumour cells, with little effect on normal cells (Alexander *et al.*, 1986; O'Neill *et al.*, 1987; Jenkins, 1989).

Misonidazole (**1**) (Figure 1) has undergone extensive clinical evaluation as a radiosensit-

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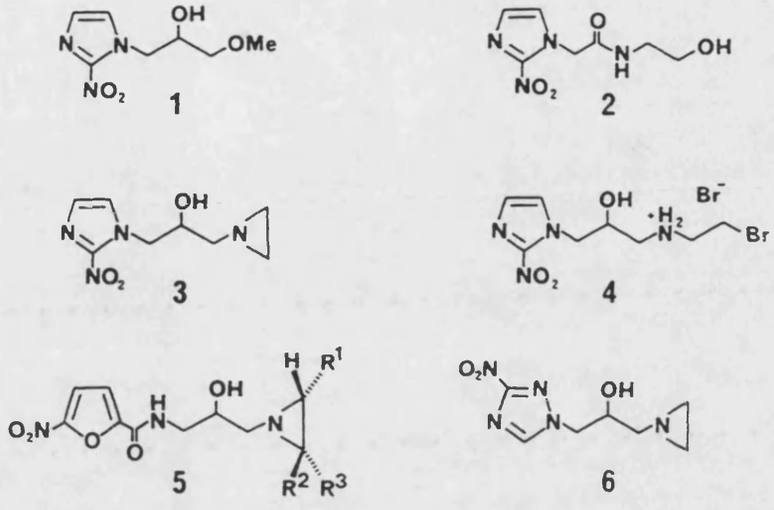


Figure 1

izer, but has failed clinically due to neurotoxicity (Adams & Stratford, 1988; Dische, 1988). The equally potent but less toxic compound, etanidazole (**2**) (Coleman *et al.*, 1984), is in phase III trials. Our approach has been to develop more potent compounds by introducing alkylating moieties, and has led to the nitroimidazoles α-[(1-aziridinyl)methyl]-2-nitroimidazole-1-ethanol (RSU-1069, **3**) (Adams *et al.*, 1984; Ahmed *et al.*, 1986) and a pro-drug designed to reduce the observed gastrointestinal toxicity of **3** (Horwich *et al.*, 1986), α[[(2-bromoethyl)amino]methyl]-2-nitroimidazole-1-ethanol (RB-6145, **4**) (Jenkins *et al.*, 1990). A large number of nitroheterocycles, both azoles and other ring systems, remain unevaluated as dual-functional radiosensitizers. We have reported recently on our investigations into compounds with higher electron affinities in nitrofurans, **5** (Naylor *et al.*, 1990) and compounds with similar electron affinities to these nitropyrazoles (nitrotriazoles, **6**) have been reported by this laboratory (Jenkins *et al.*, 1989). These studies clarified further the relationship between electron affinity, electrophilic reactivity and dual function radiosensitization but met with only limited success in the search for compounds of equal or greater efficacy than **3** whilst lowering the toxicity of this compound. Highly electron affinic 5-nitrofurans ($-E^1_7 = 250$–350 mV) were found to be highly efficient radiosensitizers *in vitro* but were shown to have high aerobic toxicity and little hypoxia-selectivity as cytotoxins. 3-Nitrotriazoles showed similar or improved radiosensitizing efficiency, in accord with their redox behaviour and significantly lower aerobic toxicity compared with the corresponding 2-nitroimidazoles but with little or no hypoxia-selective cytotoxicity. No study to date has addressed the synthesis and evaluation of the closely related aziridinyl nitroazoles with 1 or 2 nitrogen atoms, which, in their simplest, unsubstituted forms, would be expected to be of lower electron affinity than corresponding 2-nitroimidazoles, although some simple monofunctional nitropyrazoles (Asquith *et al.*, 1974), nitropyrazole hydroxamic acids (Mulcahy *et al.*, 1986), and nitropyrroles (Ruddock & Greenstock, 1977) have been studied.

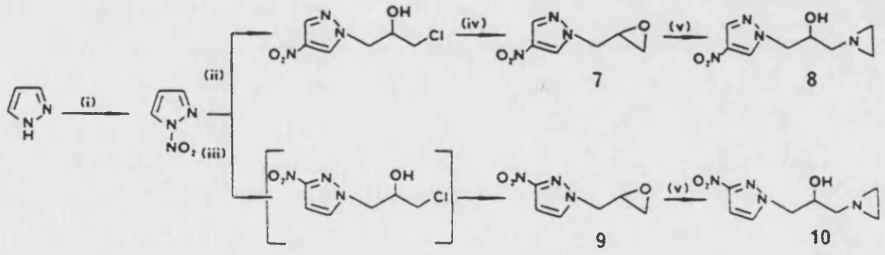
Nitropyrroles are expected to have poor electron-affinities (Ruddock & Greenstock, 1977), and correspondingly low E^1_7 values; however the redox properties of the nitropyrroles can be altered. Substitution with electron withdrawing groups and alterations to the patterns of substitution can be achieved without major synthetic difficulties, to give a series of compounds with varied E^1_7 values. We have sought to investigate nitropyrroles closely related to the parent hypoxia selective agent **3**, and as such have synthesized both C-

substituted and N-substituted 4- and 5-nitropyrroles bearing the potentially alkylating aziridinyl, substituted aziridinyl, oxiranyl and cyclopropyl groups.

We also chose to investigate the nitropyrazoles closely related to 3 which are the only other nitrogen heterocycles of this type that have not been evaluated as dual-function hypoxia-selective agents to date, although some acetohydroxamic acid derivatives have been studied (Mulcahy *et al.*, 1986). The one-electron reduction potentials of 3- and 4-nitropyrazoles were unknown prior to this study (Wardman, 1989), although half-wave reduction potentials for 1- and 2-nitropyrazole have been reported (Ruddock & Greenstock, 1977). Polarographic measurements on 4-nitropyrazole have been carried out (Rowley *et al.*, 1979), and recent studies have shown that this compound forms a comparatively stable one-electron radical which may be important in any biological effects (Edwards & Tocher, 1990).

Chemistry

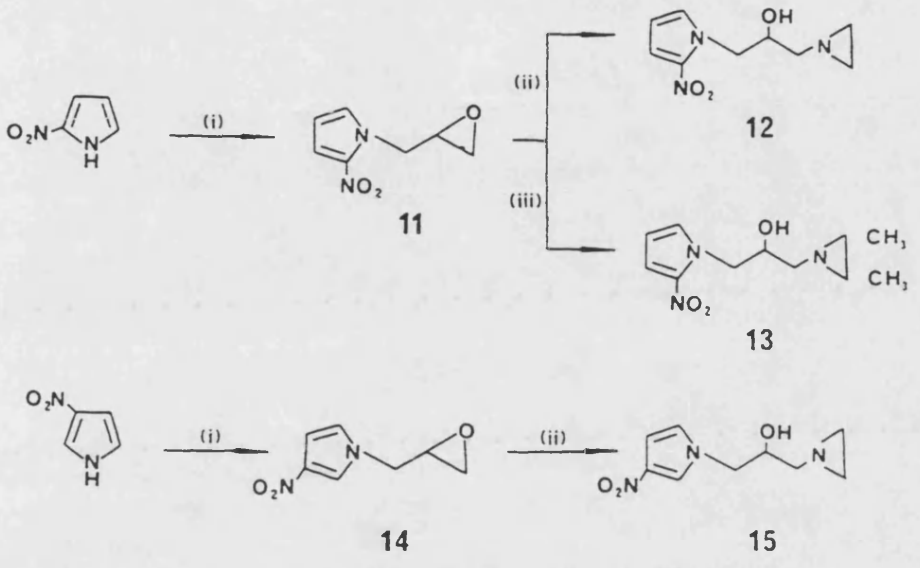
Nitration of pyrazole with acetyl nitrate in acetic acid (Huttel and Buchele, 1955) affords *N*-nitropyrazole in 68% yield. Treatment of this compound with c.H_2SO_4 affords 4-nitropyrazole, whereas heating in a high boiling solvent yields 3-nitropyrazole via a thermally induced 1,5-sigmatropic rearrangement (Janssen *et al.*, 1976). Treatment of these isomeric pyrazoles with refluxing epichlorohydrin affords the oxiranyl synthons 7 and 9 (Scheme I). In the case of the 4-nitro derivative, the chlorohydrin was isolated and required treatment with base. Treatment of these expoxides with aziridine in refluxing ethanol gave the target aziridinyl derivatives 8 and 10.



Scheme I (i) AcOH Ac_2O HNO_3. (ii) (a) c.H_2SO_4 (b) epichlorohydrin. (iii) (a) 140°C 10 h (b) epichlorohydrin. (iv) 10% NaOH(aq). (v) aziridine/Et_3N/EtOH

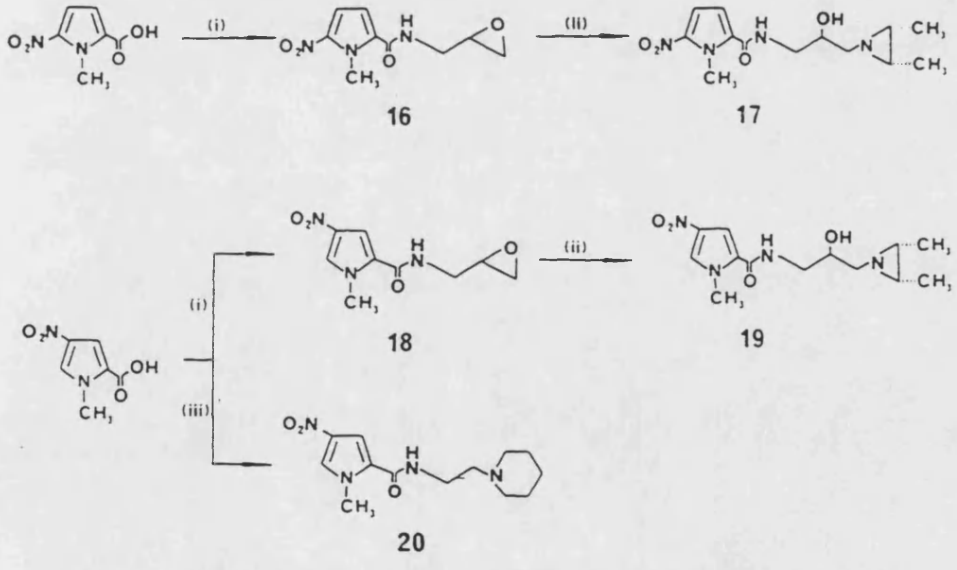
Derivatives of nitropyrroles which are close analogues of 3, although expected to be less electron affinic, were considered important targets in this study. Pyrrole can be nitrated to give predominantly 2-nitropyrrole although small quantities of the 3-nitropyrrole can be isolated by column chromatography (Morgan & Morrey, 1966). The 2- and 3-nitropyrroles were efficiently *N*-alkylated with allyl bromide via their sodium salts and subsequently converted to the oxiranes with MCPBA to afford pyrroles 11 and 14 (Scheme II). Reaction of these oxiranes with appropriate aziridines in refluxing methanol or ethanol gave the target aziridines 12, 13 and 15.

Introduction of a carboxamide moiety onto the heterocyclic ring is expected to influence the electron affinity markedly, as we have observed with a series of nitrofurans (Naylor *et al.*, 1990). Compounds of this type are available from 1-methylpyrrole-2-carboxylic acid, which was efficiently nitrated with Ac_2O/HNO_3 and the resulting 4- and 5-nitro derivatives separated as their methyl esters on silica essentially as described by Bailer *et al.* (1978).



Scheme II (i) (a) NaH/DMF/allylbromide. (b) MCPBA/CH_2Cl_2. (ii) aziridine/Et_3N/EtOH. (iii) *cis*-2,3-dimethylaziridine/Et_3N/EtOH

These esters were then hydrolysed and converted into their corresponding acyl chlorides with $SOCl_2$/THF (tetrahydrofuran). Reaction with allylamine then furnished the allyl amides which were epoxidized with MCPBA (3-chloroperbenzoic acid) (55–65% yield) to give the oxiranes **16** and **18** (Scheme III). Reaction of these derivatives with unsubstituted aziridine was unsuccesful but could be achieved with *cis*-2,3-dimethylaziridine to give



Scheme III (i) (a) $SOCl_2$/THF. (b) allylamine/CH_2Cl_2/Et_3N (c) MCPBA/CH_2Cl_2. (ii) *cis*-2,3-dimethylaziridine/Et_3N/THF. (iii) (a) $SOCl_2$/THF (b) 1-(2-aminoethyl)piperidine/THF

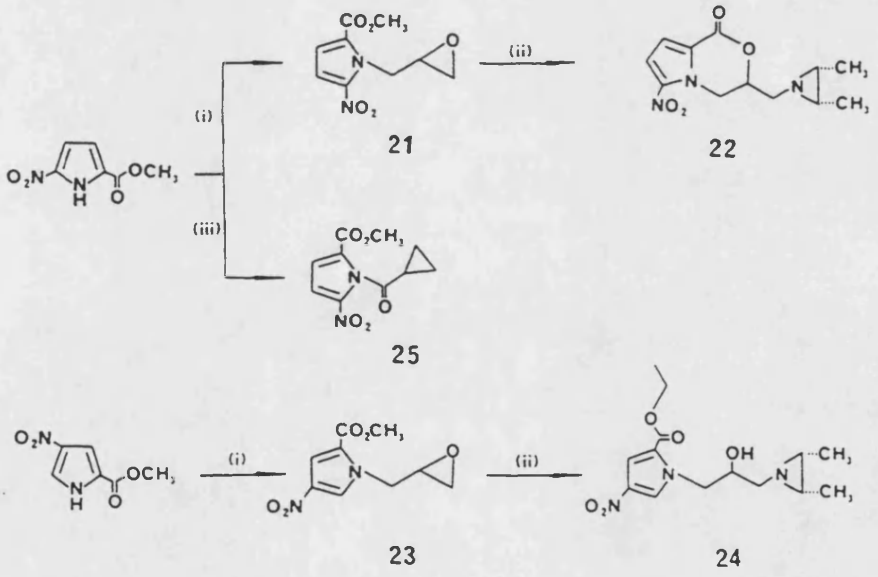
compounds **17** and **19**. A compound containing a basic rather than an alkylating side-chain, as present in the known nitroimidazole, pimonidazole, which shows selective uptake into tumours (Dische *et al.*, 1986), was synthesized by condensation of 1-(2-aminoethyl) piperidine with 1-methyl-4-nitropyrrole-2-carboxylic acid to give **20**.

A series of compounds bearing the functionalized side chain on the pyrrole nitrogen and further substitutions on the ring carbon atoms were synthesized from the common precursor pyrrole-2-carboxylic acid (Morgan & Morrey, 1971). Nitration again gives a mixture of 4- and 5-nitropyrroles separated by silica gel chromatography. Generation of the oxiranylmethyl derivatives **21** and **23** (Scheme IV) was then achieved by conversion to the sodium salt in DMF and reaction with glycidyl tosylate. Reaction of **23** with *cis*-2,3-dimethylaziridine with concurrent transesterification gave the aziridinyl analogue **24**. Reaction of **21** with *cis*-2, 3-dimethylaziridine resulted in the bicyclic fused pyrrole **22**.

The cyclopropyl carbonyl derivative **25** was synthesized by condensation of the corresponding 5-nitropyrrole-2-carboxylic ester with cyclopropyl carbonyl chloride (Scheme IV).

Synthetic chemistry

N.m.r. spectra were obtained at 60 MHz with a Jeol PMX60SI spectrometer with $SiMe_4$ as internal standard. Mass spectra were carried out on a Finnigan 4500 instrument in the electron-impact mode. Melting points are uncorrected. Tetrahydrofuran (THF) was distilled from CaH_2 and dimethylformamide was distilled at reduced pressure and stored over 4A sieves. Commercially available 3-chloroperbenzoic acid (MCPBA) was purified by washing with phosphate buffer (pH 7) and drying at reduced pressure over P_2O_5. *Cis*-2,3-dimethylaziridine was prepared as described by Dickey *et al.* (1952) and 1(H)-aziridine was purchased from Serva Fine Biochemicals Ltd. Elemental analyses were determined by Elemental Microanalysis Ltd, Okehampton, UK and Butterworth Laboratories, Teddington, UK. Where elemental analyses are indicated by symbols, analytical results obtained



Scheme IV (i) NaH/DMF glycidyl tosylate, (ii) *cis*-2,3-dimethylaziridine Et_3N/EtOH, (iii) cyclopropylcarbonyl chloride

for these elements are within ± 0.4% of the theoretical values. Solutions in organic solvents were dried by treatment with anhydrous Na_2SO_4 and filtration. Solvents were removed by evaporation under reduced pressure. All compounds were racemic. The one-electron reduction potentials (E^1_7) of all target compounds were determined as previously described (Wardman & Clarke, 1976).

α-[(1-Aziridinyl)methyl]-4-nitropyrazole-1-ethanol (8)

Pyrazole was converted to the *N*-nitro derivative with Ac_2O/HNO_3 in AcOH (Huttel & Buchele, 1955) in 87.5% yield (m.p. = 90–92°. Lit. 92–93°C). 1-Nitropyrazole (5.72 g, 0.05 mmol) was added slowly to c.H_2SO_4 (20 ml) and stirred for 20 h at ambient temperature, poured onto ice (150 g), filtered, and the filtrate extracted with Et_2O (3 × 200 ml). The combined extracts were dried and evaporated to afford 4.63 g (81%) of 4-nitropyrazole as a colourless solid (m.p. = 149–150°C). ^{1}H-n.m.r. ($CDCl_3$/$(CD_3)_2SO$ δ 8.2 (s,2H) ppm.

4-Nitropyrazole (2.26 g, 0.02 mol) and Na_2CO_3 (0.21 g, 2.0 mmol) were heated under reflux in epichlorohydrin (20 ml) for 1 h. The hot solution was then filtered and washed with hot EtOH, cooled and evaporated. The residue was taken up in EtOAc (250 ml), washed with H_2O (3 × 200 ml), sat.NaCl(aq) (200 ml), dried and evaporated. The residue was purified on silica (150 g) eluting with $CHCl_3$/MeOH (9:1) to afford 0.35 g (10.4%) 1-oxiranylmethyl-4-nitropyrazole (7) as a pale yellow oil.

^{1}H-n.m.r. ($CDCl_3$) δ 2.55 (dd, J = 2.5 and 4 Hz, 1H) and 2.9 (t, J = 4.5 Hz, 1H), oxiranyl 3-CH_2, 3.4 (m, 1H, oxiranyl 2-H), 3.95 (dd, J = 6 and 14.5 Hz, 1H) and 4.6 (dd, J = 3.6 and 14.5 Hz, 1H), arom-CH_2, 8.0 (s, 1H, pyrazole-H), 8.2 (s, 1H, pyrazole-H) ppm.

Further elution of the column afforded 1.87 g (45.5%) 1-(2-hydroxy-3-chloropropyl)-4-nitropyrazole as a pale yellow solid (m.p. = 59–61°C). ^{1}H-n.m.r. ($CDCl_3$/$(CD_3)_2SO$) δ 3.55 (dd, J = 1.5 and 4.8 Hz, 1H, C*H*$_2$Cl), 4.1–4.4 (m, 3H, arom-C*H*$_2$C*H*(OH)), 4.8 (s, 1H, O*H*), 8.0 (s, 1H, pyrazole-H), 8.35 (s, 1H, pyrazole-H) ppm.

This material was converted to the oxiranyl synthon by treatment with 100 ml 10% NaOH(aq) for 1 h at 5°C, diluting with H_2O (150 ml) and extracting with $CHCl_3$ (3 × 100 ml). The combined extracts were dried and evaporated to give a further 0.8 g (65%) of 7.

7 (0.8 g, 4.7 mmol) was dissolved in EtOH/Et_3N (99:1) (25 ml) and heated under reflux with aziridine (2.0 g, 46.5 mmol) for 30 min. Half the solution was then evaporated and, upon cooling 0.38 g (39%) of **8** was obtained as a pale yellow solid, recrystallized from EtOH/Et_3N (99:1) (m.p. = 144–145°C).

^{1}H-n.m.r. ($CDCl_3$/$(CD_3)_2SO$) δ 1.2 and 1.7 (2 × dd, J = 2 and 4 Hz, 2 × 2H, $N(CH_2)_2$), 2.3 (2 × dd, J = 4 and 7.5 Hz, 2H, C*H*$_2$-aziridine), 4.0–4.5 (m, 3H, arom-C*H*$_2$C*H*(OH), 8.0 (s, 1H, pyrazole-H), 8.5 (s, 1H, pyrazole-H) ppm.

Anal.$C_8H_{12}N_4O_3$ C, H, N.

α-[(1-Aziridinyl)methyl]-3-nitropyrazole-1-ethanol (10)

1-Nitropyrazole (5.68 g, 0.05 mol) was dissolved in xylene (100 ml) and heated at 130–140°C for 10 h. The solvent was evaporated to dryness to afford 3-nitropyrazole (3.81 g, 67.5%) as a colourless solid (m.p. = 166–167°C) which was used without further purification.

^{1}H-n.m.r. ($CDCl_3$/$(CD_3)_2SO$) δ 7.0 (d, J = 3 Hz, 1H), 7.8 (d, J = 3 Hz, 1H) ppm.

This material was treated with epichlorohydrin as described above for 7 to afford 2.3 g (40%) 1-oxiranylmethyl-3-nitropyrazole (**9**). None of the corresponding chlorohydrin was isolated in this sample.

^{1}H-n.m.r. ($CDCl_3$) δ 2.6 (dd, J = 3 and 5 Hz, 1H) and 3.0 (t, J = 5 Hz, 1H), oxiranyl 3-

CH_2, 3.45 (m, 1H, oxiranyl 2-H), 4.0 (dd, J = 6 and 14.5 Hz, 1H) and 4.6 (dd, J = 3.6 and 14.5 Hz, 1H), arom-CH_2, 7.0 (d, J = 3 Hz, 1H, pyrazole-H), 7.6 (d, J = 3 Hz, 1H, pyrazole-H) ppm.

Conversion to the target aziridine was carried out as described above for **8** to give 1.08 g (48.4%) of **10** as a pale yellow solid, recrystallized from Et_2O (m.p. = 120–121°C).

^{1}H-n.m.r. ($CDCl_3$/$(CD_3)_2SO$) δ 1.25 and 1.7 (2 × dd, J = 2 and 4 Hz, 2 × 2H, $N(CH)_2$), 2.3 (dd, J = 4 and 7.5 Hz, 2H, CH_2-aziridine), 4.1–4.5 (m, 3H, arom-CH_2CH(OH)), 6.8 (d, J = 3 Hz, 1H, pyrazole-H), 7.7 (d, J = 3 Hz, 1H, pyrazole-H) ppm.

Anal.$C_8H_{12}N_4O_3$ C, H, N.

α-[(1-Aziridinyl)methyl]-5-nitropyrrole-1-ethanol (12)

Pyrrole (10 g, 0.15 mol) was dissolved in Ac_2O (60 ml) and cooled to −20°C. HNO_3 (70%, 8 ml) in Ac_2O (38 ml) was then added slowly, maintaining the temperature at −20°C for 30 min. The reaction mixture was then poured onto ice (250 g), stirred for 30 min and extracted with Et_2O (3 × 250 ml). The solvent was then dried and evaporated and the residue purified on silica (250 g) eluting with hexane/CH_2Cl_2 (1:1, v/v) to give 3.8 g (23%) 2-nitropyrrole as pale yellow needles (m.p. = 60–61°C, lit. (Morgan & Morrey, 1966) 65–66.5°C). Further elution yielded 0.5 g (3%) of 3-nitropyrrole (m.p. = 98–99°C, lit.101°C).

2-Nitropyrrole, (1.0 g, 8.9 mmol), was dissolved in 5 ml DMF and NaH (0.47 g, 9.8 mmol) added with stirring. The solution was stirred for 30 min at room temperature and then allyl bromide (1.2 ml, *ca.*10 mmol) added with stirring. After 2 h the DMF was removed and the residue redissolved in EtOAc (75 ml) and washed with H_2O (3 × 100 ml) and sat.NaCl (100 ml), dried and evaporated. The residue was purified on silica (100 g) eluting with CH_2Cl_2 to give 1.2 g (88%) of 2-nitro-1-(prop-2-enyl)pyrrole, as a yellow oil.

T.l.c.($CHCl_3$) R_f = 0.75.

^{1}H-n.m.r. ($CDCl_3$) δ 4.8 (brd, J = 6.5 Hz, 2H, allylic-CH_2), 5.1 (m, 2H) and 5.7–6.0 (m, 1H), olefinic, 6.2 (dd, J = 1.5 and 3 Hz, 1H, pyrrole-H), 6.8 (t, J = 1.5 Hz, 1H, pyrrole-H), 7.1 (dd, J = 1.5 and 3 Hz, 1H, pyrrole-H) ppm.

This material was epoxidized as described for **16** with MCPBA and final purification on silica, eluting with hexane/CH_2Cl_2 (1:1, v/v) to afford the oxirane **11** in 37% yield as a pale yellow gum.

^{1}H-n.m.r. ($CDCl_3$) δ 2.5 (dd, J = 2.4 and 4.8 Hz, 1H), and 3.0 (t, J = 3.6 Hz, 1H), oxiranyl 3-CH_2, 3.5 (m, 1H, oxiranyl 2-H), 4.3 (dd, J = 6 and 12 Hz, 1H), 5.1 (dd, J = 2.4 and 4.8 Hz, 1H) arom-CH_2, 6.3 (dd, J = 1.5 and 3 Hz, 1H, pyrrole-H), 7.0 (t, J = 1.5 Hz, 1H, pyrrole-H), 7.3 (dd, J = 1.5 and 3 Hz, 1H, pyrrole-H) ppm.

The oxiranyl synthon **11** was used immediately and without further purification; 0.15 g (0.9 mmol) was dissolved in 2 ml EtOH (1% Et_3N) together with 0.2 g (4.6 mmol) 1(H)-aziridine. The solution was heated under reflux for 15 min, cooled and evaporated. The residual material was twice recrystallized from EtOH/Et_3N (99:1) to afford 55 mg (29%) **12** as a pale yellow solid (m.p. = 108–109°C).

T.l.c. (EtOH/Et_3N, 99:1) R_f = 0.3.

^{1}H-n.m.r. ($CDCl_3$ δ) 1.1 and 1.8 (2 × dd, J = 2 and 4 Hz, 2 × 2H, $N(CH_2)_2$), 2.2–2.5 (2 × dd, J = 4 and 7.5 Hz, 2H, CH_2-aziridine), 3.9–4.5 (m, 3H, arom-CH_2CH(OH)), 6.1 (dd, J = 1.5 and 3 Hz, 1H, pyrrole-H), 6.8 (t, J = 1.5 Hz, 1H, pyrrole-H), 7.1 (dd, J = 1.5 and 3 Hz, 1H, pyrrole-H) ppm.

Anal.$C_9H_{13}N_3O_3$ C, H, N.

α-[(cis-2,3-Dimethyl-1-aziridinyl)methyl]-5-nitropyrrole-1-ethanol (13)

11, 0.2 g (1.2 mmol) was dissolved in 2.5 ml MeOH(1% Et_3N) together with 0.2 ml (*ca.*4 mmol) *cis*-2,3-dimethylaziridine and the solution heated under reflux for 45 min. The

solution was cooled and evaporated and the residue recrystallized from EtOH to afford 0.21 g (73%) of **13** as a pale yellow solid (m.p. = 87–88.5°C).

T.l.c. (EtOAc/Et_3N, 99:1) $R_f = 0.35$.

^{1}H-n.m.r. ($CDCl_3$) δ 1.1 (d, J = 6 Hz, 6H, 2 × aziridine-CH_3), 1.5 (m, 2H, 2 × aziridine-H), 2.5 (dd, J = 2.4 and 4.8 hz, 2H, CH_2-aziridine), 4.2–4.8 (m, 4H, arom-$CH_2CH(OH)$), 6.2 (dd, J = 1.5 and 3.0 Hz, 1H, pyrrole-H), 7.0 (t, J = 1.5 Hz, 1H, pyrrole-H), 7.2 (dd, J = 1.5 and 3 Hz, 1H, pyrrole-H) ppm.

Anal.$C_{11}H_{17}N_3O_3.0.25\ H_2O$ C, H, N.

α-[(1-Aziridinyl)methyl]-4-nitropyrrole-1-ethanol (15)

1-Oxiranylmethyl-4-nitropyrrole (**14**) was prepared in 33% yield (as a similar high boiling oil) by the same route as that used for **11** using the 4-nitropyrrole available as the minor product from the nitration of pyrrole described above.

T.l.c.(hexane/CH_2Cl_2, 1:1) $R_f = 0.12$.

^{1}H-n.m.r. ($CDCl_3$) δ 2.55 (dd, J = 2.4 and 4.8 Hz, 1H) and 2.8 (t, J = 3.6 Hz, 1H), oxiranyl 3-CH_2, 3.3 (m, 1H, oxiranyl 2-H), 3.9 (dd, J = 4.8 and 14 Hz, 1H), and 4.4 (dd, J = 3 and 14 Hz, 1H), arom-CH_2, 6.6 (m, 2H, 2 × pyrrole-H), 7.6 (t, J = 1.5 Hz, 1H, pyrrole-H) ppm.

14, 0.15 g (0.9 mmol), was converted to the aziridine **15** as described for **12** to afford, after recrystallization from EtOH/Et_3N (99:1), 57 mg (30%) **15** (m.p. = 124–125.5°C).

T.l.c.(EtOH/Et_3N, 99:1) $R_f = 0.25$

^{1}H-n.m.r. ($CDCl_3$) δ 1.1 and 1.8 (2 × dd, J = 2 and 4 Hz, 2 × 2H, N-$(CH_2)_2$), 2.2–2.5 (2 × dd, J = 4 and 7.5 Hz, 2H, CH_2-aziridine), 3.9–4.6 (m, 4H, arom-$CH_2CH(OH)$), 6.7 (m, 2H, 2 × pyrrole-H), 7.6 (t, J = 1.5 Hz, 1H, pyrrole-H) ppm.

Anal.$C_9H_{13}N_3O_3$ C, H, N.

N-*(3-(cis-2, 3-Dimethyl-1-aziridinyl)-2-hydroxypropyl)-1-methyl-5-nitropyrrole-2-carboxamide (17)*

N-Methyl-5-nitropyrrole-2-carboxylic acid (0.5 g, 2.94 mmol) was dissolved in 2.5 ml THF together with 1.5 ml $SOCl_2$ and the solution heated under gentle reflux for 10 min, cooled and evaporated. The residue was redissolved in 5 ml CH_2Cl_2 and added dropwise to a solution of allylamine (1.0 g, 17.5 mmol) in CH_2Cl_2 (5 ml) containing 3 ml Et_3N. The solution was stirred at 0°C for 30 min, 20 ml H_2O added and the organic layer washed with 5% $NaHCO_3$ (50 ml), H_2O (25 ml), dried and evaporated. The residue was recrystallized from ethanol to afford 0.5 g (82%) of 1-methyl-5-nitro-*N*-(prop-2-enyl)pyrrole-2-carboxamide as a pale yellow solid (m.p. = 91–92°C).

^{1}H-n.m.r. ($CDCl_3$) δ 4.0 (brt, J = 4.8 Hz, 2H, allylic CH_2), 4.2 (s, 3H, N-CH_3), 5.1–6.0 (m, 3H, olefinic), 6.2 (brs, 1H, NH), 6.4 (d, J = 4.5 Hz, 1H, pyrrole-H), 7.1 (d, J = 4.5 Hz, 1H, pyrrole-H) ppm.

0.5 g (2.4 mmol) of the above material was dissolved in 5 ml CH_2Cl_2 together with 1.0 g (5.8 mmol) MCPBA and the solution heated under gentle reflux for 30 min; 10% Na_2SO_3 (25 ml) was then added and the organic layer washed with the same solution followed by 5% $NaHCO_3$ (2 × 25 ml) and H_2O (25%ml). The solution was dried, evaporated and purified on silica (100 g) eluting with $CHCl_3$ to afford, after recrystallization from ethanol, 0.3 g (56%) of **16** as a white solid (m.p. = 94–95°C).

T.l.c. ($CHCl_3$) $R_f = 0.2$.

^{1}H-n.m.r. ($CDCl_3$) δ 2.7 (dd, J = 2.4 and 4.8 Hz, 1H,) and 2.9 (t, J = 3.6 Hz, 1H), oxiranyl 3-CH_2, 3.3 (m, 1H, oxiranyl 2-H), 3.65 (dd, J = 4.8 and 12 Hz, 1H) and 3.9 (dd, J = 4 and 12 Hz, 1H), $CONHCH_2$, 4.3 (s, 3H, N-CH_3), 6.4 (d, J = 4.5 Hz, 1H, pyrrole), 7.1 (d, J = 4.5 Hz, 1H, pyrrole-H) ppm.

Anal.$C_9H_{11}N_3O_4$ C, H, N.

The oxiranyl synthon **16** above (0.25 g, 1.13 mmol) was dissolved in 4 ml dry THF (1% Et_3N) together with 0.2 g (2.8 mmol) *cis*-2,3-dimethylaziridine and heated under reflux for 2.5 h, cooled, evaporated and the residue recrystallized from CH_2Cl_2 to afford 0.175 g (68%) of **17** as a pale yellow solid (m.p. = 101–102°C).

^{1}H-n.m.r. ($CDCl_3$) δ 1.2 (d, J = 6 Hz, 6H, 2 × aziridine-CH_3), 1.4 (m, 2H, 2 × aziridine-H), 2.3 (dd, J = 5 and 13 Hz, 1H) and 2.5 (dd, J = 5 and 13 Hz, 1H), $CHCH_2$-aziridine, 2.7 (brs, 1H, CH(O*H*)), 3.4–3.8 (m, 3H, $NHCH_2CH(OH)$), 4.2 (s,3H,N-CH_3), 6.5 (d, J = 4.5 Hz, 1H, pyrrole-H), 7.1 (d, J = 4.5 Hz, 1H, pyrrole-H) ppm.

Anal.$C_{13}H_{20}N_4O_4$ C, H, N.

N-(3-(cis-2,3-Dimethyl-1-aziridinyl)-2-hydroxypropyl)-1-methyl-4 nitropyrrole-2-carboxamide (19)

The corresponding 4-nitro oxiranyl synthon **18** was prepared as described for the above 5-nitropyrroles from 1-methyl-4-nitropyrrole-2-carboxylic acid in 37% overall yield (m.p. = 167–168°C).

T.l.c. (EtOAc) R_f = 0.4.

^{1}H-n.m.r. ($(CD_3)_2SO$) δ 2.6 (dd, J = 2.4 and 4.8 Hz, 1H) and 2.8 (t, J = 3.6 Hz, 1H), oxiranyl 3-CH_2, 3.2 (dd, J = 4.8 and 12 Hz, 1H) and 3.5 (dd, J = 4 and 12 Hz, 1H), $CONHCH_2$, 4.0 (s, 3H, N-CH_3), 7.5 (d, J = 1.5 Hz 1H, pyrrole-H), 7.8 (d, J = 1.5 Hz, 1H, pyrrole-H), 8.5 (brs, 1H, NH) ppm.

This material was then converted to the *cis*-2,3-dimethylaziridinyl analogue **19** in 39% yield as described above for **17** (m.p. = 139–140°C).

^{1}H-n.m.r. ($CDCl_3$) δ 1.2 (d, J = 6 Hz, 6H, 2 × aziridine-CH_3), 1.5 (m, 2H, 2 × aziridine-H), 2.35 (dd, J = 4.8 and 12 Hz, 1H) and 2.7 (dd, J = 4.8 and 12 Hz, 1H), CH_2-aziridine, 3.0–3.8 (m, 4H, $NHCH_2CH(OH)$), 3.95 (s, 3H, N-CH_3), 7.0 (d, J = 1.5 Hz, 1H, pyrrole-H), 7.4 (d, J = 1.5 Hz, 1H, pyrrole-H) ppm.

Anal.$C_{13}H_{20}N_4O_4$ C, H, N.

1-Methyl-4-nitro-N-(2-(piperidin-1-yl)ethyl)pyrrole-2-carboxamide hydrochloride (20)

1-Methyl-4-nitropyrole-2-carbonyl chloride (2.0 g, 11 mmol, prepared as described above), was dissolved in 20 ml dry CH_2Cl_2 and added to a solution of 1-(2-aminoethyl)piperidine (2.0 g, 14 mmol) in CH_2Cl_2 (10 ml) and Et_3N (10 ml) at 0°C. After 15 min the solution was washed with sat.$NaHCO_3$ (2 × 50 ml) and H_2O (50 ml), dried and evaporated. The residue was redissolved in 10 ml CH_2Cl_2 and 5 ml of a solution of 1.0 M HCl in Et_2O added dropwise at 0°C. The resulting solid was filtered off and washed with Et_2O to afford **20** (1.9 g, 80%) as a white solid (m.p. = 246–247°C (dec.)).

^{1}H-n.m.r. (D_2O) δ 1.8–1.9 (m, 6H, $NCH_2(CH_2)_3CH_2N$), 3.4–3.8 (m, 8H, $NHCH_2CH_2N$- and piperidine CH_2NCH_2), 3.9 (s, 3H,N-CH_3), 7.2 (d, J = 1.5 Hz, 1H, pyrrole-H), 7.85 (d, J = 1.5 Hz, 1H, pyrrole-H) pp.

Anal.$C_{13}H_{21}N_4O_3Cl.H_2O$ C, H, N.

3-((cis-2,3-Dimethyl-1-aziridinyl)methyl)-1-oxo-3,4-dihydro-6-nitro-1-H-pyrrolo[2,1-c] oxazine (22)

Methyl 5-nitropyrrole-2-carboxylate (1.0 g, 5.9 mmol) was dissolved in 10 ml DMF and 0.2 g (6.5 mmol) NaH (80% in oil) added with stirring. The solution was stirred at 20°C for 30 min and glycidyl tosylate (prepared according to Klunder *et al.*, 1986) added (1.5 g, 6.6 mmol) and stirring continued for 15 h. The solvent was then removed and the residue

redissolved in 50 ml $CHCl_3$ and washed with H_2O (3 × 150 ml), dried and evaporated. The residue was purified on silica (200 g) eluting with CH_2Cl_2 to afford, after recrystallizing from ethanol, 0.56 g (42%) methyl 1-(oxiranylmethyl)-5-nitropyrrole-2-carboxylate (**21**) as pale yellow needles (m.p. = 56–57°).

T.l.c. (CH_2Cl_2) $R_f = 0.3$.

^{1}H-n.m.r. ($CDCl_3$) δ 2.45 (dd, J = 2.4 and 4.8 Hz, 1H) and 2.8 (t, J = 3.6 Hz, 1H), oxiranyl 3-CH_2, 3.3 (m, 1H, oxiranyl 2-H), 3.9 (s, 3H, N-CH_3), 5.2 (d, J = 4 Hz, 2H, arom-CH_2), 6.9 (d, J = 4.5 Hz, 1H, pyrrole-H), 7.1 (d, J = 4.5 Hz, 1H, pyrrole-H) ppm.

Anal. $C_9H_{10}N_2O_5$ C, H, N.

The oxiranyl synthon **21** above (0.1 g, 0.44 mmol) was dissolved in 2.5 ml MeOH-(1% Et_3N) together with 0.1 g (1.4 mmol) *cis*-2,3-dimethylaziridine. The solution was then heated under reflux for 1.5 h, cooled and evaporated. The residue was recrystallized twice from ethanol (1% Et_3N) to give 0.85 g (73%) of **22** as a pale yellow solid (m.p. = 109–110°C).

T.l.c. (EtOAc/Et_3N, 99:1) $R_f = 0.58$.

^{1}H-n.m.r. ($CDCl_3$) δ 1.1 (d, J = 6 Hz, 6H, 2 × aziridine-CH_3), 1.4 (m, 2H, 2 × aziridine-H), 2.6 (dd, J = 2.4 and 4.8 Hz, CHCH_2-aziridine), 4.2–4.9 (m, 4H, arom-CH_2CH(OH)), 6.9 (d, J = 4.5 Hz, 1H, pyrrole-H), 7.1 (d, J = 4.5 Hz, 1H, pyrrole-H) ppm.

MS, m/z 266 $(M+H)^+$ (100), 110, 85.

Anal. $C_{12}H_{15}N_3O_4 \cdot 0.25\ H_2O$ C, H, N.

*Ethyl 1-(3-(*cis*-2, 3-dimethyl-1-aziridinyl)-2-hydroxypropyl)-4-nitropyrrole-2-carboxylate (**24**)*

The oxiranylmethyl synthon **23** was prepared in 45% yield as described for **21** but using methyl 4-nitropyrrole-2-carboxylate as starting material (m.p. = 85–86°C).

T.l.c. (CH_2CL_2) $R_f = 0.24$.

^{1}H-n.m.r. ($CDCl_3$) δ 2.5 (dd, J = 2.4 and 4.8 Hz, 1H) and 2.9 (t, J = 3.6 Hz, 1H), oxiranyl 3-CH_2, 3.4 (m, 1H, oxiranyl 2-H), 2.9 (s, 3H, CO_2CH_3), 4.2 (dd, J = 6 and 14 Hz, 1H) and 5.0 (dd, J = 2.5 and 14 Hz, 1H), arom-CH_2, 7.4 (d, J = 1.5 Hz, 1H, pyrrole-H), 7.65 (d, J = 1.5 Hz, 1H, pyrrole-H) ppm.

This oxiranyl intermediate 0.5 g (2.2 mmol) was dissolved in 10 ml ethanol (1% Et_3N) together with 0.5 g (7 mmol) *cis*-2,3-dimethylaziridine and the solution heated under reflux for 2 h, cooled and evaporated. The residue was recrystallized from ethanol (1% Et_3N) to afford 0.4 g (59%) of **24** as a pale yellow solid (m.p. = 74–75°C).

T.l.c. (EtOAc/Et_3N, 99:1) $R_f = 0.5$.

^{1}H-n.m.r. ($CDCl_3$) δ 1.1 (dd, J = 6 Hz, 6H, 2 × aziridine-CH_3), 1.4 (t, J = 6.5 Hz, 3H, CO_2CH_3), 1.5 (m, 2H, 2 × aziridine-H) 2.4 (dd, J = 2.4 and 4.8 Hz, 2H, CH_2-aziridine), 3.6 (brs, 1H, CH (OH)), 4.1 (m, 1H, CH (OH)), 4.3 (q, J = 6.5 Hz, 2H, $CO_2CH_2CH_3$), 4.35 (dd, J = 4.8 and 12 Hz, 1H) and 4.5 (dd, J = 7.5 and 12 Hz, 1H), arom-CH_2, 7.4 (d, J = 1.5 Hz, 1H, pyrrole-H), 7.8 (d, J = 1.5 Hz, 1H, pyrrole-H) ppm.

Anal. $C_{14}H_{21}N_3O_5 \cdot 0.25\ H_2O$ C, H, N.

*Methyl 5-nitro-1-(cyclopropylcarbonyl)pyrrole-2-carboxylate (**25**)*

Methyl 5-nitropyrrole-2-carboxylate, 0.4 g (2.35 mmol), was dissolved in 3 ml CH_2Cl_2 containing 2 ml Et_3N. The solution was cooled to 0°C and cyclopropylcarbonyl chloride (0.25 g, 2.5 mmol) added with stirring. After 15 h at room temperature 20 ml CH_2Cl_2 was added and the solution washed with 25 ml 10% $NaHCO_3$, 25 ml H_2O, dried and evaporated. The residue was purified on silica, eluting with CH_2Cl_2 to give, after recrystallization from EtOH, 0.175 g (31%) of **25** as a pale yellow solid (m.p. = 67–68°C).

T.l.c. (CH_2CL_2) $R_f = 0.8$.

^{1}H-n.m.r. ($CDCl_3$) δ 1.5 (m, 5H, 5 × cyclopropyl-H), 4.0 (s, 3H, CO_2CH_3), 6.8 (d, J = 4.5 Hz, 1H, pyrrole-H), 7.1 (d, J = 4.5 Hz, 1H, pyrrole-H) ppm.

Anal.$C_{10}H_{10}N_2O_2$ C, H, N.

Biological methods

The *in vitro* radiosensitization studies were carried out as described previously with use of Chinese hamster V79–379 A cells (Adams *et al.*, 1976). Briefly, cells maintained in suspension culture were plated onto 6 cm glass Petri dishes in Eagles' minimal essential medium (MEM) containing 10% fetal calf serum and allowed to attach for 2 h. The medium was then replaced with 2 ml of a solution of the agent in phosphate-buffered saline (PBS) at pH 7.4 and the Petri dishes placed into gas-tight dural vessels. Hypoxia was induced by purging the vessels with N_2 (< 10 ppm O_2) for 1 h prior to irradiation with ^{60}Co γ-rays at a dose rate of 8 Gy/min. The drug solution was removed after irradiation and replaced with fresh medium. The cells were incubated at 37°C for 7 days before assaying for colony formation (Adams *et al.*, 1976), the plating efficiency achieved for untreated cells was routinely > 90%. In these experiments, the maximum concentrations of agents tested were such that the plating efficiency of unirradiated cells was reduced by no more than 50% after a contact time of 2 h in hypoxia at room temperature. Values of enhancement ratio, *ER* (ratio of survival curve slope, drug treated *versus* control) were obtained from single survival points (usually between 2×10^{-2} and 10^{-1}) obtained by appropriate choice of radiation dose and by assuming an unchanged extrapolation number (Adams *et al.*, 1979). The term $C_{1.6}$, which is the concentration of agent required to give an *ER* of 1.6, is used as the measure of sensitizing efficiency.

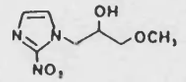
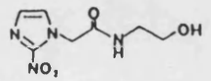
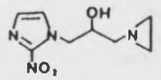
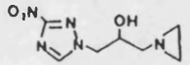
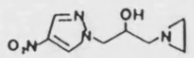
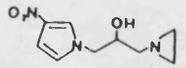
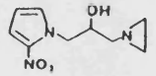
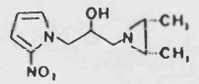
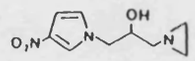
The determination of toxicity of agents to aerobic and hypoxic V79–379 A cells was carried out by using a modification of the MTT assay (Stratford & Stephens, 1989). The basis of this assay is the ability of viable cells to convert MTT into a water insoluble formazan product. Following addition to DMSO the optical density of the coloured product can be read on a multiwell spectrophotometer. Optical density is proportional to the number of surviving, metabolizing cells. In order to compare the cytotoxic efficiency of any drug we have chosen the concentration required to reduce OD to 50% of the control value from an untreated cell population. Cells were treated with drugs for 3 h at 37°C under hypoxic or aerobic conditions, then following removal of drug the cells were allowed to proliferate for 3 days, prior to MTT assay. This method for measuring bioreductive drug activity gives very similar results to those obtained using conventional clonogenic assays (Stratford & Stephens, 1989; Stratford *et al.*, 1990).

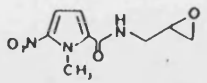
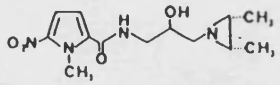
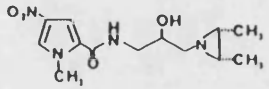
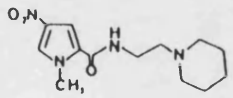
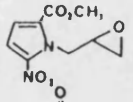
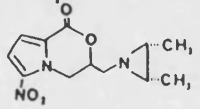
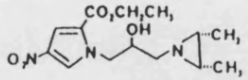
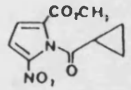
Results and discussion

A wide range of one-electron reduction potential (E^1_7) was observed (Table I) within this series of compounds, the most electron-affinic compounds being the 5-nitropyrroles ($-E^1_7$ = 326–590 mV) and the least electron-affinic compounds the 4-nitropyrroles ($-E^1_7$ = 584–657 mV). Within these two classes of compounds, those bearing carbonyl substituents had the highest E^1_7 values. The two nitropyrazoles **8** and **10** had different electron affinities, and reflects the influence of the position of the nitro group in these compounds.

These trends in redox behaviour were generally reflected in the observed radiosensitizing efficiencies although there were some exceptions. A comparison of the regression lines obtained for these compounds and the relationship between E^1_7 and $C_{1.6}$ established by Adams *et al.* (1976, 1979) is shown in Figure 2. The figure shows that all compounds

Table 1 Physicochemical and *in vitro* biological data of nitroazoles

Structure	*Compound no.*	$-E^1$, *(mV)*	*Hypoxic cell radiosensitization* $C_{1.6}$ *(mM)*[a]	C_{10}^b *Air (mM)*	C_{10}^b N_2 *(mM)*	*D. tox*[c] $\frac{C_{10}\,(Air)}{C_{10}\,(N_2)}$	*QTI*[d] $\frac{C_{10}\,(Air)}{C_{1.6}}$
	1	389	0.62[e]	45.0	4.0	**11.3**	72.6
	2	388	0.54	32.0	1.6	**20.0**	59.3
	3	389[d]	0.1	0.3	0.008	**38.0**	3.0
	6	c.a.310–340[g]	0.18[g]	0.72[g]	0.22[g]	**3.3**	4.0
	8	558[h]	0.37	0.28	0.18	**1.6**	0.75
	10	456[i]	0.2	0.15	0.085	**1.8**	0.75
	12	581[h]	0.5	0.5	0.5	**1.0**	1.0
	13	590[h]	2.0	2.6	1.2	**2.2**	1.3
	15	657[h]	0.7	0.35	n.d.	**n.d.**	0.5

	**16**	499[b]	*ER* = 1.4 (*a* 0.2[k]	0.14	0.09	**1.6**	n.d.
	**17**	491[b]	0.3	0.7	0.6	**1.2**	2.3
	**19**	609[b]	0.5	0.35	0.2	**1.8**	0.7
	**20**	602[b]	*ER* = 1.4 (*a* 0.5[k]	1.5	1.5	**1.0**	n.d.
	**21**	n.d.	*ER* = 1.2 (*a* 0.1[k]	0.18	0.18	**1.0**	n.d.
	**22**	470[b]	0.3	0.7	0.2	**3.5**	2.3
	**24**	584[b]	*ER* = 1.4 (*a* 1.0[k]	> 0.5	> 0.5	**1.0**	n.d.
	**25**	326[l]	0.17	0.4	0.12	**3.5**	2.35

n.d. = not determined

[a]$C_{1.6}$ = molar concentration of the compound required to give a sensitizer enhancement ratio (*ER*) of 1.6. Hence the lower the value of $C_{1.6}$ the greater is the sensitizing efficiency. [b]C_{40} (air or N_2) = concentration required to reduce optical density by 50% in the MTT assay (proportional to cell killing, (Stratford & Stephens, 1989)). [c]D. Tox = differential cytotoxicity. [d]Adams *et al.* (1984). [e]Adams *et al.* (1979). [f]Dennis *et al.* (1985). [g]E_1^7 Values are based upon closely related compounds (Jenkins *et al.*, 1989) other data are taken from the same reference. [h]Referred to the normal hydrogen electrode (NHE) using V30 (7,8-dihydrodipyrido-[1,2-a:2',1'-c] [1,4]diazepinedium dibromide, Homer & Tomlinson, 1960; Anderson & Patel, 1984) as redox couple; E^1, $[V30^{2+}/V30^{\bullet+}] = -549$ mV. [i]Using methyl viologen (MV) as redox couple; E^1, $[MV^{2+}/MV^{\bullet+}] = -448$ mV. [j]Using benzyl viologen (BV) as redox couple; E^1, $[BV^{2+}/BV^{\bullet+}] = -370$ mV. [k]Toxic at higher concentrations. [l]QTI = qualitative therapeutic index

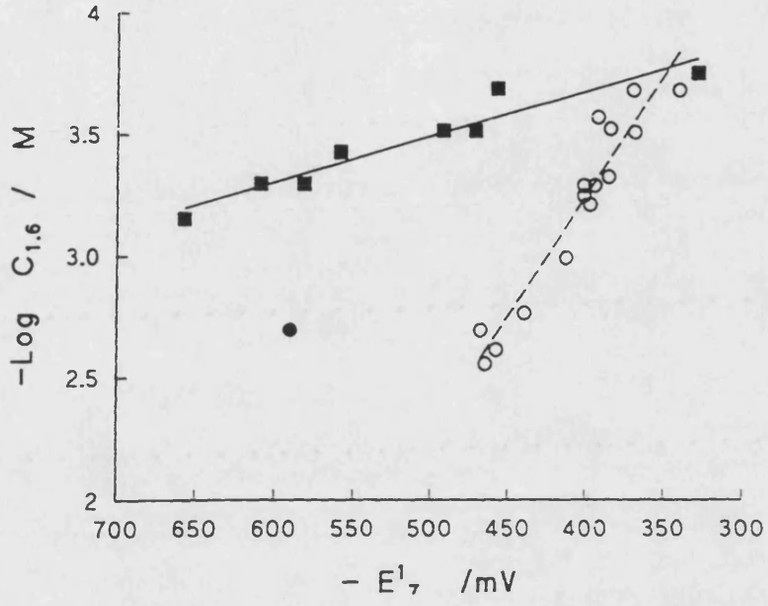


Figure 2 Dependence of sensitizing efficiency ($C_{1.6}$) on the one-electron reduction potential (E^1_7) of nitropyrazoles and nitropyrroles (■) compared with data from some monofunctional nitroheteroarenes (Adams *et al.*, 1976, 1979) (○). Compound **13** (●) was omitted from the regression

evaluated were displaced to the left relative to the established regression line, signifying enhancement in excess of that due to electron affinity. A change in the slope also indicates that large changes in E^1_7 within this series have a lesser effect on their sensitizing efficiencies than expected for monofunctional nitroheteroarenes. Only compound **13** was exceptional in its biological properties, indicating that substitution of the aziridine in the simpler pyrrole examples may have a larger influence on sensitization. All compounds except **13** and **24** are more efficient than both misonidazole (**1**) and etanidazole (**2**) and the sensitizing efficiencies of this series of nitropyrroles are generally comparable with those of similar nitrotriazoles reported previously (Jenkins *et al.*, 1989), despite their E^1_7 values being generally some 200 mV lower. Furthermore, the presence of an alkylating function has a greater effect on sensitization by nitropyrroles than has previously been observed with 2-nitroimidazoles. For example the aziridinyl 5-nitropyrrole **12** is four-fold more efficient as a radiosensitizer than is the less reactive alkylating agent, the *cis*-2,3-dimethylaziridinyl analogue **13**. This is in contrast to the differences that were observed between the corresponding nitroimidazoles, in which a 1.25-fold increase in activity is apparent with the substituted aziridine (Ahmed *et al.*, 1986). A comparison between compound **19** and its piperidinyl analogue **20**, reveals that the alkylating compound **19** is the more potent sensitizer (as shown by an *ER* of 1.6 over an *ER* of 1.4 at equal concentrations) despite previous studies (Jenkins *et al.*, 1989) showing basic substituents to give enhanced sensitizing efficiencies. The influence of alkylating reactivity, and consequently also the toxicity, upon sensitizing efficiency is therefore much greater in this series of compounds, although the unexpectedly high aerobic toxicity of the oxiranes **16** and **21** indicate that other structural features lead to general toxicity in pyrroles of this type. Nitropyrazole **10**

($E^1_7 = -456$ mV) was three-fold more efficient than **1** despite a 67 mV difference in their E^1_7 values, and the pyrazoles in this study were also at least ten-fold more efficient than were some monofunctional nitropyrazoles reported previously (Asquith *et al.*, 1974). Incorporation of alkylating groups into these compounds is expected to increase to some extent their sensitizing efficiencies (Ahmed *et al.*, 1986; Jenkins *et al.*, 1989). These levels of sensitization, however, indicate that the relationships between radiosensitization and redox behaviour observed for nitroimidazoles are not broad principles that can be applied as accurately to the nitropyrroles and nitropyrazoles. This applies in particular to compounds containing unsubstituted aziridines such as **10** and **12**.

Large changes in electron affinity were achieved with the introduction of electron withdrawing groups into the pyrrole ring and in particular the *N*-acylated derivative **25** is significantly more oxidizing. This compound is approaching the efficacy of **3** but with an E^1_7 some 50 mV more positive. The presence of the carboxyl group in the *N*-functionalized pyrroles produced a 120 mV increase in E^1_7 (compounds **13** and **22**) and at least 6-fold increases in efficiencies of radiosensitization, but with little effect upon their bioreductively-activated cytotoxicity.

All 4- and 5-nitropyrroles have lower aerobic cytotoxicities than **3**. There were no obvious relationships between aerobic toxicity and E^1_7 value within this series of agents although they were generally considerably more toxic than were some monfunctional nitroaromatic compounds with E^1_7 values of between -450 and -500 mV that have been investigated previously (Adams *et al.*, 1980). This further illustrates the extraordinary influence of the alkylating functions within these series. The closest analogues of **3** (**17**, and **19**) had similar aerobic cytotoxicities, but showed little difference in their anaerobic toxicities. It therefore appears that, as with nitrotriazoles, both nitropyrazoles and nitropyrroles are either not metabolized in a bioreductive manner or that the bioreduction products are not toxic in the same way as nitroimidazole metabolites. The aerobic toxicities, with the exception of the oxiranes, generally corresponded with the expected alkylating reactivities of the side-chain and what little additional bioreductive toxicity was present resulted in differential toxicities of up to 3.5, which is comparable to similar nitrotriazoles reported previously. The *N*-acylated derivative **25**, was one of the better dual-functioning compounds despite its weakly alkylating side-chain, and this may be attributable to its acylating potential, although we have not studied its reactivity or stability in solution.

Despite pyrroles **22** and **25** showing some hypoxia selectivity in their cytotoxic action and up to a two-fold lower aerobic toxicity than **3** there seems to be little potential for the development of nitroazoles similar to those reported here as bioreductively-activated cytotoxins. In contrast, for radiosensitization there is more promise, and we have shown that effective radiosensitization *in vitro* can be achieved with compounds of much lower (more negative) E^1_7 than previously anticipated.

We have demonstrated the effective manipulation of the electron-affinity of an otherwise poorly oxidizing nitroazole, namely nitropyrrole, resulting in the ability to produce 'tailored' compounds of the desired E^1_7 range which can be effective radiosensitizers *in vitro*. This could be adapted to the development of compounds which have also demonstrated hypoxia-selectivity such as nitroimidazoles and some nitrofurans.

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PUBLICATION 47

Synthesis of a Series of Nitrothiophenes with Basic or Electrophilic Substituents and Evaluation as Radiosensitizers and as Bioreductively-Activated Cytotoxins

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Synthesis of a Series of Nitrothiophenes with Basic or Electrophilic Substituents and Evaluation as Radiosensitizers and as Bioreductively Activated Cytotoxins

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A series of 2- and 3-nitrothiophene-5-carboxamides bearing *N*-(ω-aminoalkyl) side chains has been prepared by treatment of the thiophenecarbonyl chloride with the appropriate (protected) ω-aminoalkylamine. Analogous *N*-(oxiranylmethyl)nitrothiophene-5-carboxamides have been synthesized by epoxidation of the corresponding *N*-allylamide. Compounds in both classes were evaluated in vitro both as radiosensitizers of hypoxic mammalian cells and as selective bioreductively activated cytotoxins. The most potent radiosensitizers were those agents with strong tertiary amine bases or oxiranes in the side chain. Studies in vivo showed that 2-methyl-*N*-[2-(dimethylamino)ethyl]-3-nitrothiophene-5-carboxamide caused slight radiosensitization of the KHT sarcoma in mice given 0.34 mmol kg^{-1}. However, administration of this and related tertiary amines at higher doses was precluded by systemic toxicity.

The relative resistance of cells in hypoxic regions of solid tumors to killing by ionizing radiation remains an important reason for failure of local control of cancer by radiotherapy since molecular oxygen is required as an electron acceptor for the manifestation of damage to DNA. Electron-affinic nitroheterocycles can, however, act as mimics of molecular oxygen in this process and thus can be effective as radiosensitizers of hypoxic cells.[1,2] Indeed, a correlation between the one-electron reduction potential (E^1_7) of such compounds and their efficiency as sensitizers of hypoxic cells in vitro to ionizing radiation has been reported.[3,4]

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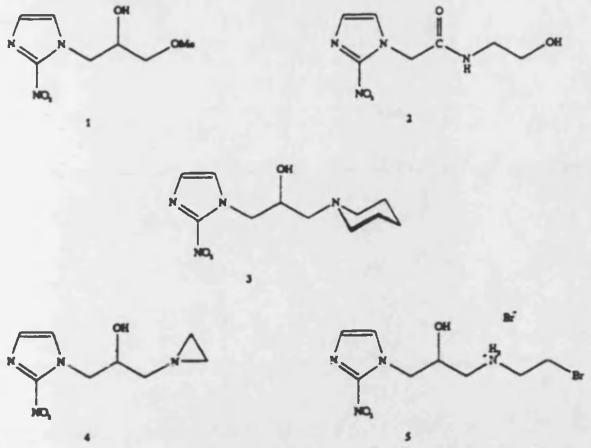
0022-2623/91/1834-2112$02.50/0

Chart I. Structures of 2-Nitroimidazole Radiosensitizers Misonidazole (1), Etanidazole (2), Pimonidazole (3), RSU 1069 (4), and RB 6145 (5)



The first such electron-affinic radiosensitizer to receive extensive clinical study was misonidazole (1, Chart I); however, owing to neurotoxicity, it was found[5] not to be possible to administer a sufficient dose of this compound to achieve radiosensitization of tumors. The second generation of agents to be the subjects of clinical trials were also 1-substituted-2-nitroimidazoles. Etanidazole (SR 2058, 2)[6,7] was designed to avoid the neurotoxicity by being highly polar and thus less able to cross the "blood–brain barrier". Pimonidazole (Ro 03-8799; 3),[8,9] which bears a basic tertiary amine, was designed to be selectively taken up into acidic regions of tumor tissue. RSU 1069 (4),[10-12] a "dual-function" agent with electrophilic and electron-affinic groups, is more potent as a radiosensitizer in experimental systems but elicited dose-limiting gastrointestinal toxicity in the clinic. RB 6145 (5),[13,14] a prodrug of 4, has recently been developed in an attempt to alleviate this problem.

Many of the 1-substituted-2-nitroimidazoles are also selectively toxic toward hypoxic cells.[15] The basis of this selective cytotoxicity is that the 2-nitroimidazole is bioreduced in these cells to an electrophile. The nature of this electrophile is still the subject of investigation, although the corresponding 2-nitrosoimidazoles and 2-(hydroxylamino)imidazoles have been implicated.[16-18] For example, 1 has been reported[19] to be 11 times more toxic toward V79 cells incubated under nitrogen than toward those incubated under air. Aziridinyl compound 4 is even more potent in this differential toxicity, the corresponding ratio[19] being 67. This potentiation is due to conversion from a monofunctional to a bifunctional electrophile upon bioreduction, which is capable[20] of cross-linking and cleaving the strands of DNA.

Recently, we have reported the synthesis and biological activity of two other series of nitroheterocycles (nitrotriazoles[21] and 2-nitrofuran-5-carboxamides[19]) augmented with basic and/or electrophilic (alkylating) side chains. In this paper, the extension of this program of drug development through the synthesis and biological evaluation of a series of 2-nitrothiophene-5-carboxamides and 2-methyl-3-nitrothiophene-5-carboxamides with electrophilic and with basic side chains is presented.

Chemical Synthesis

For the preparation of the series of nitrothiophene-2-carboxamides with the nitro group in the β-position of the heterocyclic ring, it was necessary to direct the nitration by blocking with an alkyl group the remaining α-position of a thiophene-2-carboxylic acid synthon. To this end, 2-methylthiophene-5-carboxylic acid (6) was prepared from 2-methylthiophene by Friedel–Crafts acetylation and subsequent haloform reaction, generally according to the method of Hartough.[22] Nitration with nitric acid/acetic anhydride at low temperature then gave the central intermediate 7 (Scheme I).

For the preparation of nitroheterocycles with basic amino groups in the side chains, it was necessary to couple a range of (protected) ω-aminoalkylamines with the appropriate nitroheterocyclecarboxylic acids. Formation of acid chloride 8 and subsequent treatment with the corresponding amines furnished ω-(tertiary)aminoalkylamides 9–13 in moderate to good yields after separation from highly colored byproducts and conversion to the hydrochloride salts (Scheme I). The deep colorations may be due to a small amount of nucleophilic ring opening of the nitrothiophene analogous to those reported by Dell'Erba et al.[23,24] ω-(Primary)alkylamino amide 19 was synthesized from N_α-BOC-lysine (14) in five steps. Protection of the ϵ-amine as the FMOC derivative was achieved with fluoren-9-ylmethyl chloroformate, giving 15, and the carboxylic acid was blocked as the methyl ester to give the

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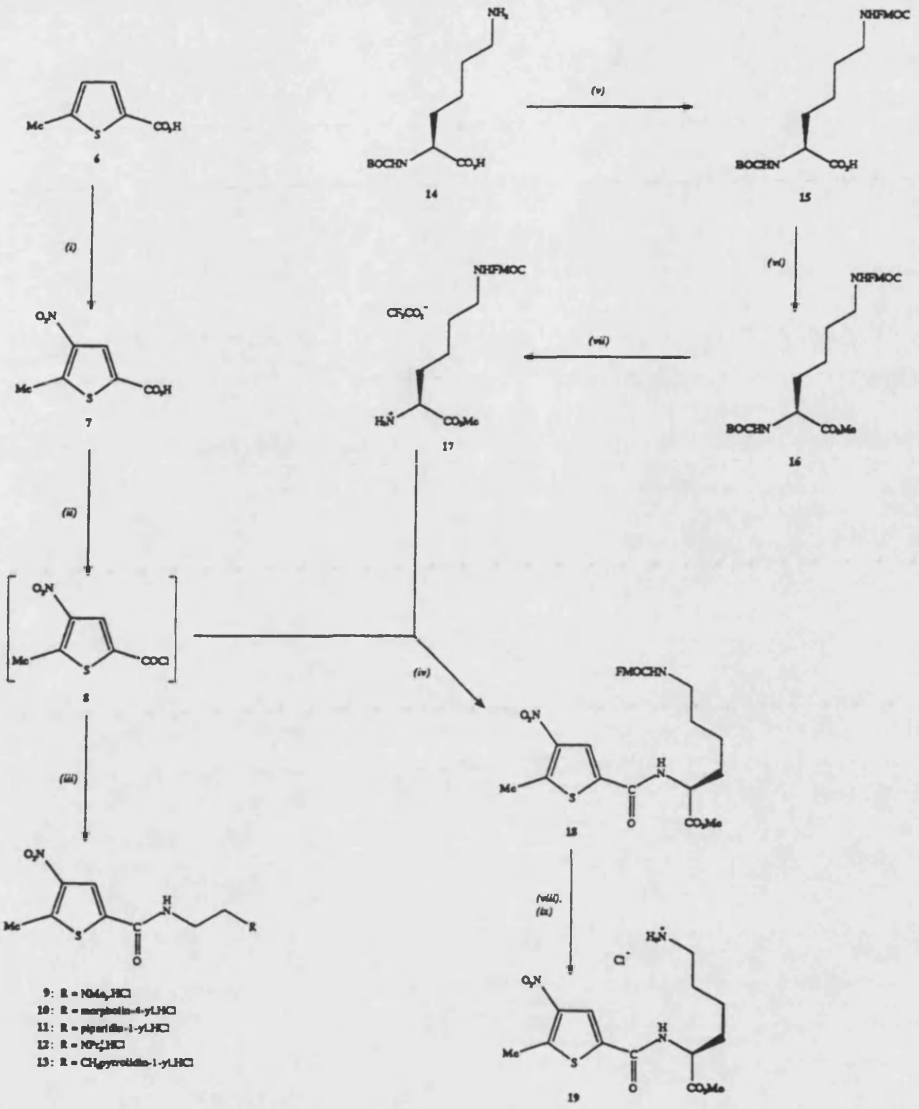
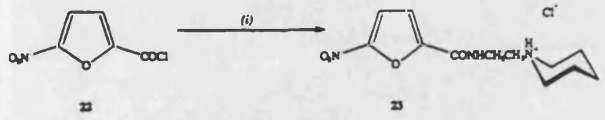
Scheme I.[a] Synthesis of 3-Nitrothiophenes 9–13 and 19 with Basic Side Chains



[a] **Reagents:** (i) HNO_3/Ac_2O; (ii) $SOCl_2$; (iii) $RCH_2CH_2NH_2/Et_2O$; (iv) Na_2CO_3/CH_2Cl_2; (v) FMOCCl/$NaHCO_3$/dioxane/H_2O; (vi) CH_2N_2/Et_2O; (vii) CF_3CO_2H; (viii) piperidine; (ix) HCl. BOC = *tert*-butoxycarbonyl. FMOC = (fluoren-9-ylmethoxy)carbonyl.

Scheme II.[a] Synthesis of Basic Nitrofuran 23

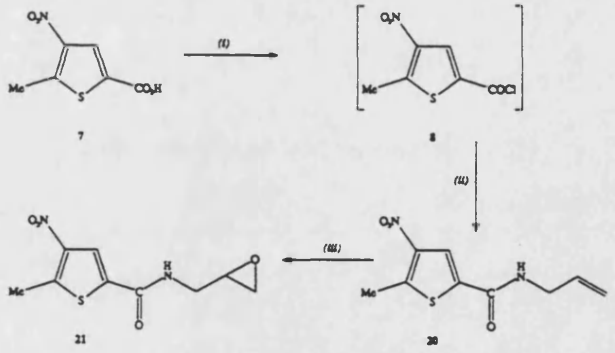


[a] **Reagents:** (i) 1-(2-aminoethyl)piperidine/Et_3N/CH_2Cl_2.

fully orthogonally protected amino acid **16**. Deprotection of the α-amino group with trifluoroacetic acid rapidly afforded the salt of amine **17**, which was coupled with **8** under mildly basic conditions, giving **18**. The required ω-aminoalkylamide **19** was then obtained by facile deprotection of the terminal amine effected by the secondary amine piperidine. In a similar manner to the general coupling, 2-nitrofuran-5-carbonyl chloride (**22**), prepared as described previously,[19] was treated with *N*-(2-aminoethyl)piperidine to yield the required nitrofuran with a basic side chain (**23**) (Scheme II).

Synthesis of nitrothiophenecarboxamides with electrophilic oxirane side chains was achieved through the corresponding *N*-allylamides, as shown in Schemes III and

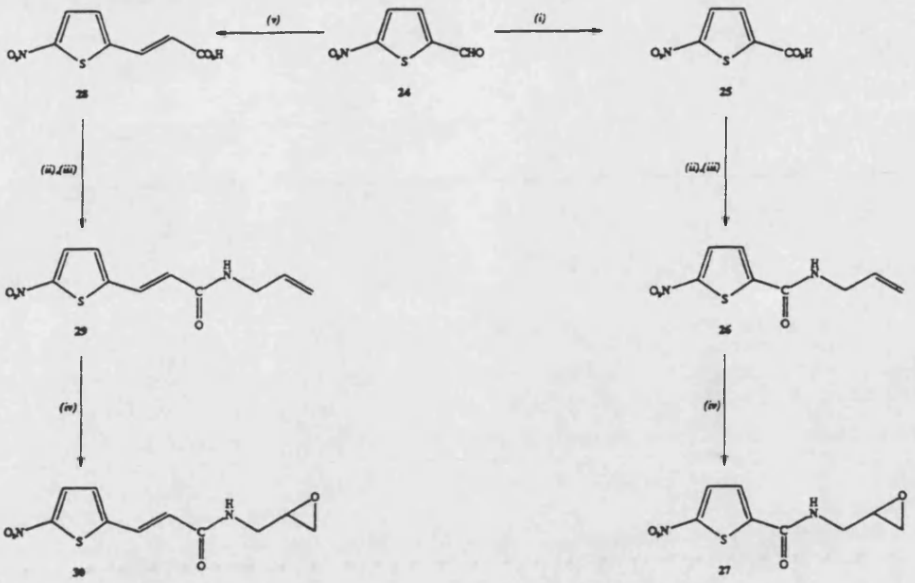
Scheme III.[a] Synthesis of 3-Nitrothiophene 21 with an Electrophilic Side Chain



[a] **Reagents:** (i) $SOCl_2$, (ii) allylamine/Et_2O, (ii) 3-chloroperoxybenzoic acid/CH_2Cl_2.

IV. Reaction of acid chloride **8** with allylamine gave 3-nitro amide **20** in excellent yield, without the attendant discoloration noted during the syntheses of the free bases

Scheme IV.[a] Synthesis of *N*-(Oxiranylmethyl)-2-nitrothiophenamides **27** and **30**

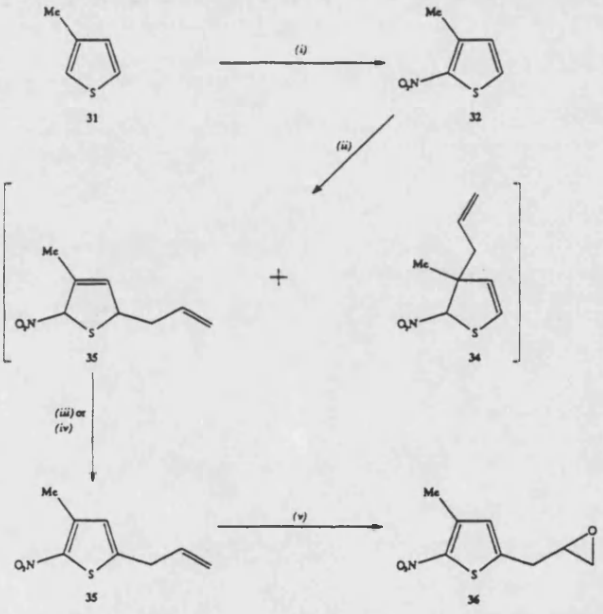


[a] Reagents: (i) $AgNO_3/H_2O$; (ii) $SOCl_2$; (iii) allylamine/CH_2Cl_2; (iv) 3-chloroperoxybenzoic acid/CH_2Cl_2; (v) malonic acid/pyridine.

of the tertiary amine amides **9–13**. Epoxidation was effected using 3-chloroperoxybenzoic acid, giving oxiranylmethylamide **23** (Scheme III). In the 2-nitro series, the commercially available 2-nitrothiophene-5-carboxaldehyde **24** was oxidized smoothly to the corresponding carboxylic acid **25** with silver(I) nitrate (Scheme IV). Treatment of **24** with potassium permanganate under a variety of conditions gave **25** in only trace amounts, in contrast to the reported[25] synthesis of **25** from 5-methyl-2-nitrothiophene with this reagent. Reaction of the corresponding acid chloride with allylamine and subsequent epoxidation with the same peroxyacid yielded allylamide **26** and oxiranylmethylamide **27**, respectively. Nitrothiophenepropenoic acid **28** was formed in moderate yield by Doebner condensation of aldehyde **24** with malonic acid in hot pyridine in a modification of the procedure of Tirouflet et al.[26] Again, the acid chloride reacted smoothly with allylamine (forming **29**). As expected, epoxidation under acidic conditions occurred only at the relatively electron-rich terminal olefin, affording the *N*-oxiranylmethyl unsaturated amide **30**.

The coupling of an appropriately functionalized three-carbon side chain to a nitrothiophene could be accomplished via conjugate addition of a Grignard reagent to an appropriate substrate and oxidative rearomatization of the intermediate addition product. Ballini et al.[27] have recently reported on the addition of a variety of alkylmagnesium halides to unsubstituted 2-nitrothiophene, noting that the products of 2,3- and 2,5-addition were both formed in each case and were separated only with great difficulty, if at all. The yields were reported to be modest. On the basis of this information, we predicted that addition of allylmagnesium bromide to 3-methyl-2-nitrothiophene (**32**) may be governed, at least in part, by steric factors

Scheme V.[a] Conjugate 2,3-Addition of a Grignard Reagent to Nitrothiophene **32**



[a] Reagents: (i) HNO_3/Ac_2O; (ii) allylmagnesium bromide/THF; (iii) 5,6-dichloro-2,3-dicyanobenzoquinone/THF; (iv) BF_3-Et_2O/THF; (v) 3-chloroperoxybenzoic acid/CH_2Cl_2. THF = tetrahydrofuran.

tending to favor formation of the product (**33**) of 2,5-addition, rather than isomer **34**. Furthermore, isolation of the required 2,3,5-trisubstituted thiophene **35** was facilitated by the inability of **34** to be oxidatively rearomatized with dichlorodicyanobenzoquinone (DDQ), owing to the presence of a quarternary carbon atom at C-3 (Scheme V). The ^1H NMR spectrum of the crude product mixture showed the presence of dihydrothiophene **34** and aromatic thiophene **35**. The latter compound was isolated easily from this mixture by chromatography in modest overall yield. Interestingly, the oxidative rearomatization was effected with equal efficiency by air in the presence of catalytic Lewis acid (boron trifluoride diethyl etherate),

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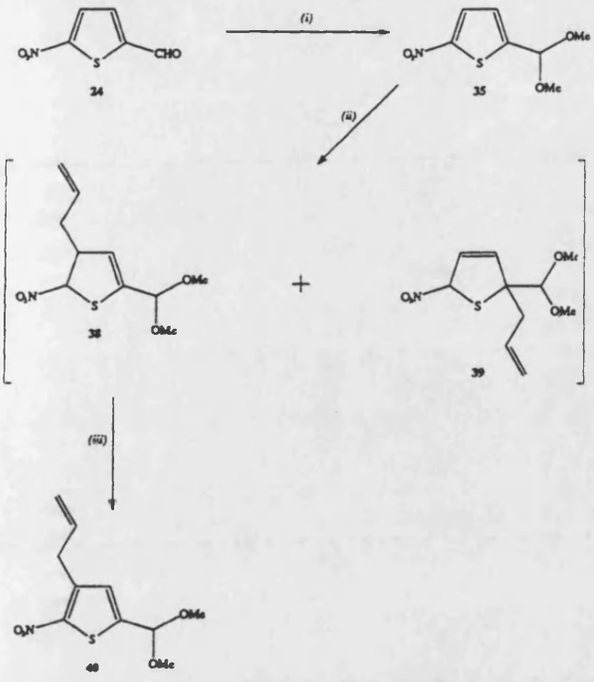
Scheme VI.[a] Conjugate 2,5-Addition of a Grignard Reagent to Nitrothiophene **36**



[a] **Reagents:** (i) MeOH/HCl; (ii) allylmagnesium bromide/THF; (iii) 5,6-dichloro-2,3-dicyanobenzoquinone/THF. THF = tetrahydrofuran.

obviating the need to remove the organic byproduct dichlorodicyanohydroquinone. Epoxidation with peroxyacid furnished oxiranylmethylnitrothiophene **36**. The usefulness of addition of appropriate Grignard reagents into substituted 2-nitrothiophenes was demonstrated in the synthesis of compound **40** (Scheme VI), which requires selective addition in the 2,3-mode, rather than in the 2,5-mode as above. Protection of the aldehyde function of **24** was carried out by formation of dimethyl acetal **37**. In this substrate, the dimethoxymethyl group should have a large steric requirement and direct addition toward the 2,3-mode. Addition of allylmagnesium bromide to **37** gave a mixture of dihydrothiophenes **38** and **39**. Of these, only **38** could be rearomatized with DDQ, giving **40** as the only thiophene product, albeit in poor yield. Attempted epoxidation of **40** failed owing to acid-catalyzed degradation of the acetal function.

Although strict analogues of lead compound **4**, bearing an aziridinyl group, were sought, treatment of oxiranes **21**, **27**, **30**, and **36** with aziridine gave only complex, highly colored mixtures. Similarly, reaction of these oxiranes with substituted aziridines 2,2-dimethylaziridine and cis-2,3-dimethylaziridine, which gave more stable adducts in the nitrofuran series,[19] also failed to furnish identifiable, stable products.

The one-electron reduction potentials (E^1_7) were measured, according to the method previously described by us,[11] for compounds **9–11**, **13**, **23**, **26**, **27**, **30**, and **36**. Amides of 2-methyl-3-nitrothiophene-5-carboxylic acid (**7**) are some 200–250 mV less oxidizing than those of 2-nitrothiophene-5-carboxylic acid (**25**); this trend is similar to that observed for related nitrofurans.[19] Interestingly, Breccia et al.[30] have recently reported values of redox potential for 5-nitrothiophenes, which are in broad agreement with those reported in this study. However, the value reported[30] for a close analogue of **7** (a 4-nitrothiophene) is at variance with those determined here for a range of 4-nitrothiophenes.

Table I. Radiosensitization by, and Redox Potentials of, Nitrothiophenes **9–13**, **19**, **21**, **23**, **26**, **27**, **30**, **36**, and **40** and Nitrofuran **23** in Vitro (Data for nitroimidazoles **1**, **4**, and **5** are shown for comparison.)

compd	$C_{1.6}$,[a] mM	ER max[b] (concn, mM)	E^1_7,[c] mV
1	0.6[d]	2.6 (10)[e]	−389[e]
4	0.1[f]	3.1 (0.5)[d]	−389[f]
5	0.1[d]	3.1 (0.5)[d]	
9	0.1	2.0 (0.5)	−499
10	1.0	1.6 (1.0)	−537
11	0.07	3.2 (1.0)	−537
12	0.07	2.2 (0.2)	
13	0.03	2.5 (0.5)	−519
19	0.05	1.6 (0.05)	
21	0.2	1.6 (0.2)	
23	0.01	1.9 (0.05)	−242
26	0.08	1.6 (0.08)	−272
27	0.05	1.9 (0.1)	−271
30	0.05	1.6 (0.05)[h]	−274
36	0.06	2.7 (0.1)	−481
40		1.3 (0.005)	

[a] Concentration of compound required to give enhancement ratio = 1.6. [b] Enhancement ratio at maximum concentrations of compounds (in parentheses). This maximum concentration was the highest concentration which did not cause significant cytotoxicity to the cells. [c] Referred to the normal hydrogen electrode (NHE), using methyl viologen, benzyl viologen, and triquat as redox couples ($E^1_7[V^{2+}/V^{\cdot+}]$ = −448, −370, and −549 mV, respectively).[28] [d] Data taken from ref 13. [e] Data taken from ref 4. [f] Data taken from ref 29. [g] Data taken from ref 11. [h] Concentration limited by solubility.

Biological Evaluation in Vitro

Nitrothiophenes **9–13**, **19**, **21**, **26**, **27**, **30**, **36**, and **40** and nitrofuran **23** were assayed for their ability to sensitize hypoxic V79 cells in vitro to γ-radiation, as described previously.[13,19] The results are expressed in Table I as ER max, the maximum factor (enhancement ratio) by which the cells were sensitized to radiation when compared to control cells without added nitroheterocycles, and $C_{1.6}$, the concentration of nitroheterocycle required to give an enhancement ratio of 1.6.

The 2-methyl-3-nitrothiophene-5-carboxamides with primary or tertiary amine bases in the side chains, compounds **9**, **11–13**, and **19**, all have values of $C_{1.6}$ in the range 0.03–0.1 mM. The exception is morpholinyl analogue **10**, which would be expected[31] to be markedly less basic than **9**, **11–13**, and **19**, owing to the presence of the heterocyclic oxygen; this compound gives $C_{1.6}$ = 1.0 mM. The structurally analogous *N*-oxiranylmethylcarboxamide **23**, with a potentially electrophilic rather than a basic side chain, is intermediate in potency.

For the strong tertiary amine bases **9** and **11–13**, significant toxicity toward the experimental cells was not observed at concentrations equal to $C_{1.6}$ and higher values of the enhancement ratio (up to 3.2 for **11**) were achieved at concentrations up to 1 order of magnitude higher than $C_{1.6}$. The remaining, less potent compounds, **10** and **21**, together with primary amine **19**, elicited significant killing of cells through toxicity at concentrations equal to $C_{1.6}$. Thus 2-methyl-3-nitrothiophene-5-carboxamides **9** and **11–13** are markedly more potent than is misonidazole (**1**)

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Table II. Selective Toxicity of Nitrothiophenes 10–12, 19, 21, 26, 27, and 30 and Nitrofuran 23 to Hypoxic V79 Cells in Vitro (Data for nitroimidazoles 1, 4, and 5 are shown for comparison.)

compd	C_{50}(AIR),[a] mM	$C_{50}(N_2)$,[a] mM	ratio[b]
1	45.0[c]	4.0[c]	11[c]
4	0.3[c]	0.0045[c]	67[c]
5	2.3[d]	0.09[d]	26[d]
10	2.5	2.5	1.0
11	1.8	0.8	2.3
12	0.5	0.25	2.0
19	0.35	0.35	1.0
21	0.35	0.4	0.9
23	0.05	0.016	3.0
26	0.30	0.11	2.5
27	0.15	0.15	1.0
30	0.08	0.08	1.0
36	0.065	0.045	1.3

[a] C_{50}(AIR) and $C_{50}(N_2)$ are the concentrations of compounds required to reduce the optical density by 50% compared to controls when the incubations in the MTT assay are performed under air or under nitrogen, respectively;[41] optical density is proportional to the number of viable cells present at the end of the period of incubation. [b] Ratio = C_{50}(AIR)/$C_{50}(N_2)$. [c] Data taken from ref 19. [d] These data are the concentrations required to reduce the survival of cells to 1% in a clonogenic assay and are taken from ref 13.

and, indeed, equal or greater in potency than is the leading 2-nitroimidazole with an electrophilic side chain, compound 4, despite having redox potentials (E^1_7) some 100 mV more negative (less oxidizing) than those of the 2-nitroimidazoles. These data are in apparent contrast to the relationship[3,4] between increasing radiosensitizing potency in vitro and more positive redox potential found for a wide range of nitro(hetero)aromatic agents. An apparent enhancement of radiosensitizing potency has, however, been reported[32-34] for electron-affinic compounds with strong organic bases in the side chain. This phenomenon has been ascribed[35-38] to enhanced uptake of these bases by cells in culture, leading to higher effective concentrations within the cell compared to those in the external medium. In addition, weak bases analogous to those described here are reported[39,40] to be able to concentrate adjacent to DNA through electrostatic interactions between the negatively charged DNA phosphate backbone and the positively charged conjugate acids of the amino groups on the sensitizers. This effect may well be operating for 9, 11–13, and 19 but not for the less basic morpholine 10. To investigate whether this method of increasing potency could be applied to a nitroheterocycle of more positive redox potential, 2-nitrofuran-5-carboxamide 23 was evaluated for radiosensitizing efficiency and

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Table III. Maximum Tolerated Ip Doses of Nitrothiophenes 9–11, 13 and 19 and Nitrofuran 23 in C3H/He Mice (Data for nitroimidazoles 1, 4, and 5 are shown for comparison.)

compd[a]	MTD (ip),[b] mmol kg^{-1}	compd[a]	MTD (ip),[b] mmol kg^{-1}
1	4.00[c]	11	<0.34
4	0.38[c]	13	0.24
5	1.00[c]	19	0.34
9	0.34	23	0.17
10	0.34		

[a] Compounds were dissolved in 0.1 M phosphate-buffered saline solution at pH 7.4 immediately before use and were injected in a total volume equivalent to 0.02 mL per g mouse body weight. [b] The maximum tolerated dose (MTD) is the highest single dose which did not produce severe or persistent clinical signs or death of the adult non-tumor-bearing mice within 24 h. [c] Data taken from ref 14.

Table IV. Radiosensitization by Nitrothiophene 9 and Nitrofuran 23 of KHT Tumors in Vivo (Data for nitroimidazole 4 are shown for comparison.)

compd	optimum time,[a] min	dose,[b] mmol kg^{-1}	surviving fraction[c]
none			2.8×10^{-2} ($3.1–2.4 \times 10^{-2}$)
4	60	0.38	8.3×10^{-4} ($7.2–9.5 \times 10^{-4}$)
9[d]	30	0.34	5.0×10^{-3} ($2.9–8.5 \times 10^{-3}$)
23[d]	60	0.17	2.2×10^{-3} ($1.9–2.7 \times 10^{-3}$)

[a] Optimum time interval between injection of the compound and irradiation of the tumor. [b] Compounds were administered ip as in Table III, footnote *a*. [c] Surviving fraction of clonogenic cells (mean ± SE) after treatment with compound and X-rays (10 Gy). [d] Compounds 9 and 23 had no effect on the survival of clonogenic cells when irradiation was omitted.

was found to be 7-fold more potent than its analogue 11 in terms of $C_{1.6}$ (Table I). This furan has E^1_7 = –242 mV and, like other nitrofurancarboxamides,[19] is up to 270 mV more electron-affinic than the 2-methyl-3-nitrothiophene-5-carboxamides. Analogous 2-nitrofuran-5-carboxamides with potentially electrophilic or unreactive side chains have values of $C_{1.6}$ in the range 0.02–0.1 mM,[19] compared with $C_{1.6}$ = 0.01 mM for 23.

In contrast with the considerable potency of many of the compounds tested as radiosensitizers of hypoxic cells in vitro, no useful selective toxicity toward hypoxic V79 cells was shown by 3-nitrothiophenes 10–12, 19, and 21, nitrofuran 23, and 2-nitrothiophenes 26, 27, and 30 (Table II). Although the values of C_{50}(AIR), the concentration required to kill 50% of the aerobic cells under the conditions of the assay, range from 0.05 mM for the highly oxidizing nitrofuran 23 to 2.5 mM for nitrothiophene 10, little additional toxicity as a result of bioreduction is evident. Ratios of C_{50}(AIR)/$C_{50}(N_2)$ range from 0.9 to 3.0 with no apparent structure–activity relationship. Similar results have been reported by us[21] with a series of nitrotriazoles and the lack of differential toxicity toward hypoxic cells was attributed either to lack of bioreduction or to lack of chemical reactivity of the reduced products leading to lack of toxic effects. For comparison, even the monofunctional 2-nitroimidazole lead compound misonidazole (1) gives a corresponding ratio of 11, whereas "dual function" agents 4 and 5 show even higher selectivities in cytotoxicity toward hypoxic cells.

Biological Evaluation in Vivo

Selected compounds (nitrothiophenes 9–11, 13, and 19 and nitrofuran 23) were administered in escalating doses to mice by the ip route. The maximum single, nontoxic doses (MTD) were determined, initially to provide a basis for subsequent experiments on radiosensitization in vivo. The values of MTD are shown in Table III. The nitro-

thiophenes were generally not well-tolerated and at the MTD doses (0.24–0.34 mmol kg^{-1}) caused rapid respiration and bizarre stretching activity in the mice, which persisted from about 2 to 90 min after injection. At higher doses, immobility, tremors, and spasms were noted, and the mice did not recover. Nitrofuran 23 was also toxic, leading to convulsions and death after doses greater than 0.17 mmol kg^{-1}. Effects on the central nervous system are known to be caused by some tertiary amines. For example, valproic acid (2-propylbutanoic acid) is an anticonvulsant therapeutic drug[42] whereas the corresponding *N*-[2-(dimethylamino)ethyl]amide causes convulsions;[43] this amide has some structural analogy to 9 and 11–13.

As the compounds had been shown to have little selective toxicity toward hypoxic cells in culture, they were not evaluated as bioreductively activated cytotoxins in vivo. The radiosensitizing potencies of 9 and 23 were investigated and are compared with the effect of 4 in Table IV. The compounds were administered at their MTD to mice bearing the subcutaneous KHT sarcoma at various times before local irradiation of the tumors with 240 KV X-rays (10 Gy). The tumors were excised after 24 h and the survival of tumor cells was determined in a clonogenic assay.[14] In this experimental system, 4 reduced the surviving fraction of cells after irradiation to a value which would be expected if all the tumor cells were aerobic;[14] i.e. sensitization was equivalent to that of oxygen. Compound 9 was much less effective, producing no radiosensitization when administered 15 or 60 min before irradiation and only a small reduction in surviving fraction when the highest possible dose was administered 30 min before the dose of X-rays. In contrast, the highly electron-affinic nitrofuran 23 does show some radiosensitizing properties in vivo. However, even when administered at its MTD, it is markedly less effective than is the lead compound 4 when given at an equitoxic dose. Other nitrothiophenes were excluded from evaluation as radiosensitizers in vivo as they elicited equal or greater toxicity (measured by MTD) than did 9.

Conclusions

A series of nitrothiophenecarboxamides has been synthesized; those compounds (9, 11–13, 19) which have strong amine bases in the side chain are potent sensitizers of hypoxic cells to the effects of γ-radiation in vitro, as is nitrofuran 23. Indeed, these compounds are markedly more potent as radiosensitizers in vitro than would be predicted[3,4] from their relatively negative one-electron redox potentials. However, systemic toxicity precludes the translation of these potencies into useful antitumor radiosensitization in vivo. Similarly, general toxicity prevents the demonstration of selective bioreductively activated cytotoxicity toward hypoxic V79 cells in vitro. Although the compounds were cytotoxic to hypoxic V79 cells at concentrations equal to or 1 order of magnitude lower than for misonidazole (1), despite much more negative values of E^1_7, strong nonselective (aerobic) toxicity was manifest also at similar concentrations.

It is concluded that despite the undoubted intrinsic potency of 2-methyl-3-nitrothiophene-5-carboxamides and related compounds as radiosensitizers of hypoxic cells, development of a clinically useful agent of this type will depend on the understanding of the mechanisms of general toxicity and design of suitable analogues to circumvent this problem.

Experimental Section

NMR spectra were obtained at 60 MHz with JEOL PMX60SI and at 270 MHz with JEOL GX270 spectrometers, using $SiMe_4$ as internal standard. A Philips PU9516 instrument furnished the IR spectra, which were obtained with Nujol mulls except where indicated. Mass spectra were obtained with a VG 7070 spectrometer in either the electron impact (EI) mode at 70 eV or in the chemical ionization mode (with 2-methylpropane). Melting points are uncorrected. Tetrahydrofuran (THF) was distilled from CaH_2. Brine refers to saturated aqueous NaCl. Elemental analyses were determined by Elemental Microanalysis Ltd., Okehampton, U.K. The one-electron reduction potentials (E^1_7) of selected compounds (Table I) were determined as previously described.[11] Solutions in organic solvents were dried by treatment with anhydrous Na_2SO_4 and filtration. Solvents were removed by evaporation under reduced pressure. Radial PLC refers to centrifugally accelerated preparative-layer chromatography using a Chromatotron (TC Research, Norwich, U.K.). All chromatography was performed with silica gel as stationary phase. 2-Methyl-3-nitrothiophene-5-carboxylic acid (7),[22,44] (*E*)-3-(3-nitrothiophen-5-yl)propenoic acid (28),[45] and 3-methyl-2-nitrothiophene (32)[46] were prepared by literature methods.

2-Methyl-*N*-[2-(dimethylamino)ethyl]-3-nitrothiophene-5-carboxamide Hydrochloride (9). Carboxylic acid 7 was treated with $SOCl_2$ and with 2-(dimethylamino)ethylamine as for the preparation of 12 below to afford 9 (43%) as off-white crystals: mp 156–157 °C; IR ν_{max} 3330, 1635, 1560, 1550 cm^{-1}; NMR (free base; $CDCl_3$) δ 2.30 (2, 6 H, $N(CH_3)_2$), 2.50 (t, J = 6 Hz, 2 H, Me_2NCH_2), 2.80 (s, 3 H, thiophene CH_3), 3.50 (q, J = 6 Hz, 2 H, $NHCH_2$), 7.05 (br, 1 H, NH), 7.90 (s, 1 H, thiophene H). Anal. C, H, N.

2-Methyl-*N*-(2-morpholin-4-ylethyl)-3-nitrothiophene-5-carboxamide Hydrochloride (10). Carboxylic acid 7 was treated with $SOCl_2$ and with 4-(2-aminoethyl)morpholine as for the preparation of 12 below to afford 10 (60%) as off-white crystals: mp 259–261 °C; IR ν_{max} 3200, 3110 (w), 2600–2450, 1635, 1540 cm^{-1}; NMR (D_2O) δ 2.80 (s, 3 H, CH_3), 3.4–4.2 (m, 12 H, morpholine 2,3,5,6-H + NCH_2CH_2N), 8.10 (s, 1 H, thiophene H); MS (EI) m/z 213 (M − C_4H_9NO), 170 (M − morpholine CH_2CH_2NH), 100 (100) (morpholine CH_2); MS (CI) m/z 300 (M + 1), 270, 100 (100) (morpholine CH_2). Anal. C, H, N.

2-Methyl-3-nitro-*N*-(2-piperidin-1-ylethyl)thiophene-5-carboxamide Hydrochloride (11). Carboxylic acid 7 was treated with $SOCl_2$ and with 1-(2-aminoethyl)piperidine as for the preparation of 12 below, except that the crude material was purified by radial PLC (Et_2O then $CHCl_3$/MeOH (9:1)) to furnish 11 (40%) as buff crystals: mp 237–238 °C; IR ν_{max} 3290, 3090 (w), 2700–2550, 1670, 1550 cm^{-1}; NMR (D_2O) δ 1.6–2.1 (m, 6 H, piperidine 3,4,5-H), 2.75 (s, 3 H, CH_3), 2.9–3.3 (m, 4 H, piperidine 2,6-H), 3.4–3.9 (m, 4 H, NCH_2CH_2N), 8.05 (s, 1 H, thiophene H); MS (EI) m/z 214 (M − piperidine CH_2), 98 (100) (piperidine CH_2); MS (CI) m/z 298 (M + H), 268 (M − NO), 98 (100) (piperidine CH_2).

***N*-[2-[*N*,*N*-Bis(1-methylethyl)amino]ethyl]-2-methyl-3-nitrothiophene-5-carboxamide Hydrochloride (12).** Carboxylic acid 7 (380 mg, 2 mmol) was boiled under reflux in $SOCl_2$ (3 mL) for 10 min and the excess reagent was evaporated. (2-Aminoethyl)bis(1-methylethyl)amine (0.75 mL) was added to the residue in Et_2O (20 mL). After 10 min, the suspension was poured into CH_2Cl_2, was washed twice with saturated aqueous $NaHCO_3$, with H_2O, and with brine, and was dried. The evaporation residue, in propan-2-ol (100 mL), was treated with aqueous HCl (10 M, 0.6 mL). Recrystallization of the evaporation residue from propan-2-ol gave 12 (420 mg, 59%) as off-white crystals: mp 216–218 °C dec; IR ν_{max} 3250, 2680, 1665, 1545 cm^{-1}; NMR (D_2O) δ 1.35 (d, J = 6 Hz, 12 H, 2 × $C(CH_3)_2$), 2.75 (s, 3 H, thiophene CH_3),

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3.05 (septet, J = 6 Hz, 2 H, 2 × C*H*Me$_2$), 3.3–3.8 (m, 4 H, NCH$_2$CH$_2$N), 8.10 (s, 1 H, thiophene H). Anal. C, H, N.

2-Methyl-3-nitro-*N*-(3-pyrrolidin-1-ylpropyl)thiophene-5-carboxamide Hydrochloride (13). Carboxylic acid 7 was treated with $SOCl_2$ and with 1-(3-aminopropyl)pyrrolidine as for the preparation of 12 above to afford 13 (31%) as off-white crystals: mp 214–215 °C dec; IR ν_{max} 3240, 2580, 2500, 1650, 1540 cm^{-1}; NMR (D_2O) δ 1.2–1.7 (m, 6 H, pyrrolidine 3,4-H + NCH$_2$C*H*$_2$CH$_2$N), 2.70 (s, 3 H, CH$_3$), 3.0–3.5 (m, 4 H, NC*H*$_2$CH$_2$CH$_2$N), 8.05 (s, 1 H, thiophene H). Anal. C, H, N.

***N*$_\alpha$-(*tert*-Butoxycarbonyl)-*N*$_\epsilon$-[(fluoren-9-ylmethoxy)carbonyl]lysine Methyl Ester (16).** Fluoren-9-ylmethyl chloroformate (2.585 g, 10 mmol), in 1,4-dioxane (60 mL), was added to *N*$_\alpha$-(*tert*-butoxycarbonyl)lysine (14) (2.46 g, 10 mmol) and $NaHCO_3$ (2.1 g, 25 mmol) in H_2O (60 mL) and the mixture was stirred for 16 h before being partitioned between CH_2Cl_2 (400 mL) and aqueous H_2SO_4 (1.0 M, 150 mL). The organic phase was washed with H_2O and was dried. The solvent was evaporated to give *N*$_\alpha$-(*tert*-butoxycarbonyl)-*N*$_\epsilon$-[(fluoren-9-ylmethoxy)carbonyl]lysine (15) (4.34 g, 93%) as a gum: NMR ($CDCl_3$) δ 1.45 (s + m, 15 H, C(CH$_3$)$_3$ + lysine β,γ,δ-H), 3.20 (br q, J = 6 Hz, 2 H, lysine ϵ-H), 4.0–4.5 (m, 4 H, fluroene 9-H + fluorene CH$_2$ + lysine α-H), 5.2–5.8 (br, 2 H, 2 × NH), 7.2–7.8 (m, 8 H, fluorene 1–8-H). This acid (2.17 g, 4.64 mmol) in Et_2O (100 mL) was treated with excess CH_2N_2 in Et_2O (30 mL) for 10 min until evolution of N_2 ceased. A solution of AcOH in Et_2O (10%) was then added until no more N_2 was evolved and the yellow color was discharged. The solvent was evaporated to give 16 (2.235 g, quant.) as a white solid: mp 103–105 °C; NMR ($CDCl_3$) δ 1.45 (s + m, 15 H, C(CH$_3$)$_3$ + lysine β,γ,δ-H), 3.20 (br q, J = 6 Hz, 2 H, lysine ϵ-H), 3.70 (s, 3 H, OCH$_3$), 4.1–4.6 (m, 4 H, fluorene 9-H + fluorene CH$_2$ + lysine α-H), 4.7–5.3 (br, 2 H, 2 × NH), 7.2–7.8 (m, 8 H, fluorene 1–8-H). Anal. C, H, N.

***N*$_\alpha$-[(2-Methyl-3-nitrothiophene-5-yl)carbonyl]lysine Methyl Ester Hydrochloride (19).** Protected amino acid 16 (1.45 g, 3 mmol) was treated with CF_3CO_2H (10 mL) for 20 min before the excess reagent was evaporated. The residue (*N*$_\epsilon$-[(fluoren-9-ylmethoxy)carbonyl]lysine methyl ester 17), in CH_2Cl_2 (100 mL), was washed with saturated aqueous $NaHCO_3$ and was dried. Anhydrous Na_2CO_3 (4 g) was added, followed by 2-methyl-3-nitrothiophene-5-carbonyl chloride (8 as described above; 2 mmol). The mixture was stirred for 30 min, was washed with H_2O (twice), aqueous H_2SO_4 (0.5 M), and H_2O, and was dried. Purification of the evaporation residue by radial PLC ($CHCl_3$) gave *N*$_\epsilon$-[(fluoren-9-ylmethoxy)carbonyl]-*N*$_\alpha$-[(2-methyl-3-nitrothiophene-5-yl)carbonyl]lysine methyl ester (18) (670 mg, 60%) as a gum: NMR ($CDCl_3$) δ 1.2–2.0 (m, 6 H, lysine β,γ,δ-H), 2.70 (s, 3 H, thiophene CH$_3$), 3.15 (br q, J = 6 Hz, 2 H, lysine ϵ-H), 3.75 (s, 3 H, OCH$_3$), 4.1–4.55 (m, 4 H, fluorene 9-H + fluorene CH$_2$ + lysine *N*$_\alpha$-H), 4.70 (br q, J = 6 Hz, 1 H, lysine α-H), 5.40 (t, J = 6 Hz, 1 H, lysine *N*$_\epsilon$-H), 7.3–7.9 (m, 8 H, fluorene 1–8-H), 8.20 (s, 1 H, thiophene H). This material (670 mg, 1.2 mmol) in THF (20 mL) was treated with piperidine (5 mL) for 10 min. The evaporation residue was purified by radial PLC (silica gel, $CHCl_3$/MeOH (9:1)) and was treated with aqueous HCl (10 M, 0.2 mL) in propan-2-ol (20 mL). The solvent was evaporated to obtain 19 (160 mg, 36%) as a white solid: mp 83–84 °C; NMR (D_2O) δ 1.5–2.1 (m, 6 H, lysine β,γ,δ-H), 2.80 (s, 3 H, thiophene CH$_3$), 3.15 (br t, $J \approx 7$ Hz, 2 H, lysine ϵ-H), 3.85 (s, 3 H, OCH$_3$), 4.60 (m, 1 H, lysine α-H), 8.20 (s, 3 H, thiophene H).

2-Methyl-3-nitro-*N*-prop-2-enylthiophene-5-carboxamide (20). Compound 7 was treated with $SOCl_2$ and with allylamine as for the synthesis of 12 above, except that the treatment with HCl was omitted, to give 20 (81%) as a pale buff solid: mp 84.5–86 °C; IR ν_{max} 3320, 1625, 1535 cm^{-1}; NMR ($CDCl_3$) δ 2.80 (s, 3 H, CH$_3$), 4.05 (tt, J = 6 and 1 Hz, 2 H, NCH$_2$), 5.15 (br d, J = 11 Hz, 1 H) and 5.25 (br d, J = 16 Hz, 1 H) (C=CH$_2$), 5.90 (ddt, J = 16, 11 and 6 Hz, 1 H, C*H*=CH$_2$), 6.6 (br, 1 H, NH), 7.90 (s, 1 H, thiophene H). Anal. C, H, N.

2-Methyl-3-nitro-*N*-(oxiranylmethyl)thiophene-5-carboxamide (21). Amide 20 (1.89 g, 8.36 mmol) was boiled under reflux with 3-chloroperoxybenzoic acid (50%, 6.48 g, 18.8 mmol) in CH_2Cl_2 (100 mL) for 8 h. The solution was washed three times with aqueous Na_2SO_3 (10%), twice with saturated aqueous $NaHCO_3$, with H_2O, and with brine and was dried. Column chromatography of the evaporation residue gave 21 (650 mg, 32%) as a pale buff solid: mp 92–93 °C; IR ν_{max} 3350, 3110 (w), 1625, 1535 cm^{-1}; NMR ($CDCl_3$/CD_3OD) δ 2.70 (dd, J = 4 and 2 Hz, 1 H) and 2.80 (m, 1 H) (oxirane 3-H$_2$), 2.80 (s, 3 H, thiophene CH$_3$), 3.2–3.9 (m, 3 H, NCH$_2$CH), 8.07 (s, 1 H, thiophene H); MS (EI) m/z 242.0314 (M) ($C_9H_{10}N_2O_4S$ requires 242.0361), 224, 211, 199, 170 (100) (M – oxiranylmethyl); MS (CI) m/z 243 (100) (M + H), 170.

2-Nitro-*N*-(2-piperidin-1-ylethyl)furan-5-carboxamide Hydrochloride (23). 2-Nitrofuran-5-carbonyl chloride[19] (22) (1.50 g, 10 mmol) in CH_2Cl_2 (15 mL) was added to *N*-(2-aminoethyl)piperidine (1.0 mL, 7.5 mmol) and Et_3N (7.5 mL) in CH_2Cl_2 (50 mL) at 0 °C. Stirring continued at this temperature for a further 30 min before the solution was washed twice with saturated aqueous $NaHCO_3$ and with H_2O and was dried. The evaporation residue, in THF (10 mL), was treated with ethereal HCl (1.0 M, 5.0 mL). The solid was collected and was washed with Et_2O to afford 23 (1.93 g, 85%) as a white solid: mp 256–258 °C dec; NMR (D_2O) δ 1.6–1.9 (m, 6 H, piperidine 3,4,5-H), 3.4–3.8 (m, 8 H, piperidine 2,6-H + NCH$_2$CH$_2$N), 7.20 (d, J = 3.5 Hz, 1 H, furan 4-H), 7.30 (d, J = 3.5 Hz, 1 H, furan 3-H). Anal. C, H, N.

2-Nitrothiophene-5-carboxylic Acid (25). 2-Nitrothiophene-5-carboxaldehyde (24) (3.14 g, 20 mmol) was stirred with $AgNO_3$ (6.8 g, 40 mmol) and NaOH (3.27 g, 82 mmol) in H_2O at 5 °C for 20 min. The solution was filtered, acidified by addition of aqueous HCl, and extracted three times with Et_2O. The combined extracts were washed with H_2O and with brine and were dried. Recrystallization of the evaporation residue from Et_2O/hexane furnished 25 (1.88 g, 54%) as white crystals: mp 155–157 °C (lit.[25] mp 157–158 °C); NMR ($CDCl_3$/$(CD_3)_2SO$ 2:1) δ 7.60 (d, J = 5 Hz, 1 H, thiophene 4-H), 7.80 (d, J = 5 Hz, 1 H, thiophene 3-H), 9.75 (br, 1 H, CO_2H).

2-Nitro-*N*-prop-2-enylthiophene-5-carboxamide (26). Carboxylic acid 25 (690 mg, 4 mmol) was boiled under reflux in $SOCl_2$ (3 mL) until no acid remained (TLC of sample quenched in MeOH). Excess reagent was removed by distillation and the residue was treated with allylamine (2 mL) in CH_2Cl_2 (20 mL) for 1 H. The solution was washed twice with saturated aqueous $NaHCO_3$, with H_2O, and with brine and was dried. Purification of the evaporation residue by radial PLC (CH_2Cl_2) and recrystallization from CH_2Cl_2/hexane afforded 26 (520 mg, 62%), as white crystals: mp 110–111 °C; IR ν_{max} 3310, 1630, 1555 cm^{-1}; NMR ($CDCl_3$/CD_3OD) δ 4.00 (dt, J = 6 and 1 Hz, 2 H, NCH$_2$), 5.15 (br d, J = 11 Hz, 1 H) and 5.25 (br d, J = 15 Hz, 1 H) (C=CH$_2$), 5.75 (ddt, J = 15, 11, and 6 Hz, 1 H, C*H*=CH$_2$), 7.45 (d, J = 4 Hz, 1 H, thiophene 4-H), 7.75 (d, J = 4 Hz, thiophene 3-H); MS (EI) m/z 212.0208 (M) ($C_8H_8N_2O_3S$ requires 212.0256, 197, 166, 156 (100) (M – C_3H_6N), 110; MS (CI) m/z 213 (100) (M + H).

2-Nitro-*N*-(oxiranylmethyl)thiophene-5-carboxamide (27). Amide 26 (1.16 g, 5.5 mmol) was boiled under reflux with 3-chloroperoxybenzoic acid (85%; 2.04 g, 10 mmol) in CH_2Cl_2 (50 mL) for 6 h. The solution was washed with aqueous Na_2SO_3 (10%) (twice), H_2O, saturated aqueous $NaHCO_3$ (twice), H_2O, and brine and was dried. The evaporation residue was purified by column chromatography (CH_2Cl_2/MeOH 19:1) then by radial PLC ($CHCl_3$) to furnish 27 (540 mg, 43%) as a white solid: mp 113–114 °C dec; NMR ($CDCl_3$/CD_3OD) δ 2.65 (dd, J = 4 and 2 Hz, 1 H) and 2.85 (t, J = 4 Hz, 1 H) (oxirane 3-H$_2$), 3.25 (m, 1 H, oxirane 2-H), 3.4–4.0 (m, 2 H, NCH$_2$) 7.55 (d, J = 4 Hz, 1 H, thiophene 4-H), 7.85 (d, J = 4 Hz, 1 H, thiophene 3-H); MS (EI) m/z 228.0197 (M) ($C_8H_8N_2O_4S$ requires 228.0205).

(*E*)-3-(2-Nitrothiophene-5-yl)-*N*-(oxiranylmethyl)propenamide (30). Carboxylic acid 28 (500 mg, 2.5 mmol) was boiled under reflux in $SOCl_2$ (2.5 mL) for 15 min. Allylamine (1.0 mL) was added to the evaporation residue in CH_2Cl_2 (10 mL) at 0 °C and the mixture was stirred for 30 min. The solution was washed with saturated aqueous $NaHCO_3$ and with brine before being dried. The evaporation residue was recrystallized from CH_2Cl_2/hexane to give (*E*)-3-(2-nitrothiophen-5-yl)-*N*-(prop-2-enyl)propenamide (29) (520 mg, 87%) as an off-white solid: mp 153–154 °C; NMR ($(CD_3)_2SO$/CD_3OD) δ 4.05 (dt, J = 6 and 1 Hz, 2 H, NCH$_2$), 5.18 (br d, J = 11 Hz, 1 H) and 5.30 (br d, J = 16 Hz, 1 H) (C=CH$_2$), 5.75 (ddt, J = 16, 11 and 6 Hz, 1 H, C*H*=CH$_2$), 6.80 (d, J = 15 Hz, 1 H) and 7.70 (d, J = 15 Hz, 1 H) (thiophene CH=CH), 7.50 (d, J = 4 Hz, 1 H, thiophene 4-H), 8.15 (d, J = 4 Hz, 1 H, thiophene 3-H). This amide (460 mg, 1.93

mmol) was boiled under reflux with 3-chloroperoxybenzoic acid (85%; 1.44 g, 7 mmol) in CH_2Cl_2 (50 mL) for 8 h. The solution was washed with aqueous Na_2SO_3 (twice), saturated aqueous $NaHCO_3$ (twice), H_2O, and brine and was dried. The evaporation residue was purified by column chromatography ($CHCl_3$) to furnish **30** (240 mg, 33%) as a white solid: mp 120 °C dec; IR ν_{max} 3270, 2800–2550, 1650, 1620, 1560 cm^{-1}; NMR ($CDCl_3$/$(CD_3)_2SO$) δ 2.60 (dd, J = 4 and 2 Hz, 1 H) and 2.75 (t, J = 4 Hz, 1 H) (oxirane 3-H_2), 3.0–3.9 (m, 3 H, NCH_2CH), 6.65 (d, J = 16 Hz, 1 H) and 7.50 (d, J = 16 Hz, 1 H) (propenamide 2,3-H), 7.20 (d, J = 4 Hz, 1 H, thiophene 4-H), 7.85 (d, J = 4 Hz, 1 H, thiophene 3-H), 8.3 (br, 1 H, NH); MS (EI) m/z 254.0366 (M) ($C_{10}H_{10}N_2O_4S$ requires 254.0361).

3-Methyl-2-nitro-5-prop-2-enylthiophene (35). Method A. Allylmagnesium bromide (1.0 M in THF; 5 mL, 5 mmol) was added to **32** (710 mg, 5 mmol) in THF (20 mL) during 15 min at −50 °C. Stirring continued for a further 15 min at −50 °C. 5,6-Dichloro-2,3-dicyanobenzoquinone (1.7 g, 7.5 mmol) in THF (25 mL) was added and the solution was stirred at ambient temperature for 3 h before being poured into aqueous NH_4Cl and extracted three times with Et_2O. The combined extracts were dried. The evaporation residue was purified by column chromatography (EtOAc/hexane 1:4) and by radial PLC (EtOAc/hexane 1:5) to afford **35** (170 mg, 19%) as a colorless glass: NMR ($CDCl_3$) δ 2.55 (s, 3 H, CH_3), 3.50 (br d, J = 6 Hz, 2 H, thiophene CH_2), 5.0–6.2 (m, 3 H, $CH{=}CH_2$), 6.60 (br s, 1 H, thiophene 4-H).

Method B. Compound **32** (720 mg, 5 mmol) in THF (20 mL) was added during 30 min to allylmagnesium bromide in THF (1.0 M, 6 mL, 6 mmol) at −65 °C. After a further 30 min at this temperature, $BF_3{\cdot}Et_2O$ (1.0 mL) was added and the mixture was stirred at ambient temperature for 16 h before being poured onto aqueous NH_4Cl. The mixture was stirred for 1 h and was extracted four times with Et_2O. The combined extracts were dried. The evaporation residue was purified by column chromatography (CH_2Cl_2) to give **35** (220 mg, 24%) identical with the material described above. These materials were used for the preparation of **36** without further purification.

3-Methyl-2-nitro-5-(oxiranylmethyl)thiophene (36). Nitrothiophene **35** (250 mg, 1.4 mmol) was boiled under reflux with 3-chloroperoxybenzoic acid (55%; 2.52 g, 8 mmol) in CH_2Cl_2 (40 mL) for 12 h. The mixture was washed with saturated aqueous Na_2SO_3 (three times), saturated aqueous $NaHCO_3$ (twice), and brine before being dried. The evaporation residue was purified by radial PLC (CH_2Cl_2) to give **36** (110 mg, 41%) as a colorless gum: IR (liquid film) ν_{max} 1555 (w) cm^{-1}; NMR (270 MHz; $CDCl_3$) δ 2.58 (s, 3 H, CH_3), 2.60 (dd, J = 4.8 and 2.6 Hz, 1 H) and 2.87 (dd, J = 4.5 and 3.5 Hz, 1 H) (oxirane 3-H_2), 2.95 (dd, J = 15.8 and 6.0 Hz, 1 H) and 3.08 (dd, J = 15.8 and 4.0 Hz, 1 H) (thiophene CH_2), 3.19 (ddt, J = 6.0, 2.6, and 4.0 Hz, 1 H, oxirane 2-H), 6.76 (s, 1 H, thiophene 4-H); MS (EI) m/z 199.0297 (100) (M) ($C_8H_9NO_3S$ requires 199.0303), 182, 156, 126, 110.

5-(Dimethoxymethyl)-2-nitro-3-(prop-2-enyl)thiophene (40). 2-Nitrothiophene-5-carboxaldehyde (**24**) (314 mg, 2 mmol) was boiled under reflux with MeOH (5 mL) and ethereal HCl (1.0 M, 4 mL) for 45 min. The solvent and excess reagent were evaporated to give 5-(dimethoxymethyl)-2-nitrothiophene (**37**) (406 mg, quant.) as a colorless gum: NMR ($CDCl_3$) δ 3.40 (s, 6 H, 2 × OCH_3), 5.60 (s, 1 H, $CH(OMe)_2$), 7.00 (d, J = 4 Hz, 1 H, thiophene 4-H), 7.80 (d, J = 4 Hz, 1 H, thiophene 3-H). This acetal was treated with allylmagnesium bromide according to method A above to afford **40** (15%) as a colorless oil: IR (liquid film) ν_{max} 2950, 1500, 1330 cm^{-1}; NMR ($CDCl_3$) 3.35 (s, 6 H, 2 × OCH_3), 3.75 (br d, J = 6 Hz, 2 H, thiophene CH_2), 5.05 (br d, J = 16 Hz, 1 H) and 5.10 (br d, J = 11 Hz, 1 H) ($C{=}CH_2$), 5.50 (s, 1 H, $CH(OMe)_2$), 5.80 (ddd, J = 16, 11 and 6 Hz, 1 H, $CH{=}CH_2$), 6.85 (s, 1 H, thiophene 4-H).

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Reaction of Hetarylhydrazines with 1,1,1-Trifluoropentane-2,4-dione and Ethyl 2,4-Dioxovalerate

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Reaction of hetarylhydrazines with 1,1,1-trifluoropentane-2,4-dione and ethyl 2,4-dioxovalerate

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The reaction of 2-hydrazino-4-(4′-substitutedphenyl)thiazoles with 1,1,1-trifluoropentane-2,4-dione (**1**) gives the isomeric 5(3)-trifluoromethyl-3(5)-methyl-1-[4′-(4″-substituted phenyl)thiazol-2′-yl]pyrazoles (**3** and **4**). A similar reaction of 2-hydrazinobenzothiazoles with ethyl 2,4-dioxovalerate (**2**) furnishes the isomeric-1-(6′-H/methylbenzothiazol-2′-yl)-5(3)-ethoxycarbonyl-3(5)-methylpyrazoles (**8** and **10**). However, the reaction of 2-hydrazinobenzothiazoles with **1** gives 1-(6′-substitutedbenzothiazol-2′-yl)-5-trifluoromethyl-3-methylpyrazoles (**5**) as the sole products. The intermediate 5-hydroxy-2-pyrazolines (**6**) have been isolated and characterised. The structure of the isomeric pyrazoles have been established on the basis of their NMR (^1H and ^{13}C) spectra.

We have recently described[1] the products of the reaction of several hetarylhydrazines with 1,3-diketones as having pyrazole structure instead of isomeric diazepines or triazepines as reported by earlier investigators[2]. During the course of present investigation, it was noted that the methyl groups located at positions-3 and 5 of pyrazole moiety resonated around δ 2.3 and 2.7, respectively. The deshielding of C_5-CH_3 may be due to the lone pair effect, hydrogen bonding or ring current[3].

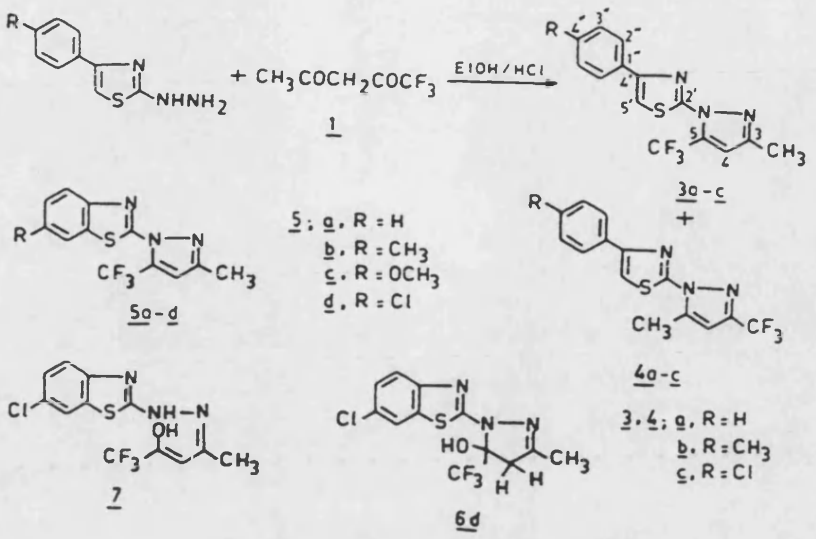
In continuation of our work on the reaction of hetarylhydrazines with 1,3-diketones, we thought it of interest to study the structures of the reaction products between hetarylhydrazines and unsymmetrical 1,3-diketones to explore the generality of this observation in distinguishing the isomeric pairs and also to determine the isomer ratio when electron withdrawing substituents such as a trifluoromethyl and ethoxycarbonyl are present. In this paper, we report the results of the reaction of hetarylhydrazines with 1,1,1-trifluoropentane-2,4-dione (**1**) and ethyl 2,4-dioxovalerate (**2**).

Condensation of 2-hydrazino-4-(4′-tolyl)thiazole with **1** furnished a mixture of two isomeric products **3b** and **4b** in the ratio 4:1 (Scheme 1) which were separated by column chromatography. The isomer whose ^1H NMR exhibited the two methyl signals around δ 2.30, was identified as **3b**. The other isomer (**4b**), obviously, has the methyl group located at position-5, which resonated characteristically at δ 2.85.

The isomers **3b** and **4b** could also be readily distinguished using ^{13}C NMR spectroscopy. The signals at δ 150.65, 111.70 and 132.20 were attributed to C-3, C-4 and C-5 carbons, respectively of the pyrazole moiety in **3b**, whereas these carbons resonated at δ 145.70, 106.54 and 142.67 in **4b**. Shielding of such a magnitude is in agreement with the observation that the replacement of methyl by a trifluoromethyl group causes an upfield shift of about 6 ppm in benzene derivatives[4]. In the case of **3b**, the CF_3 carbon signal appeared as a quartet around δ 119.52. However, the CF_3 signal could not be located in the spectra of **4b**, perhaps due to the lack of sufficient concentration of the compound. The complete assignment of all other carbon atoms was made by DEPT technique (*vide* experimental).

The other isomers (**3a**, **3c** and **4a**, **4c**) were obtained by a similar reaction of 2-hydrazino-4-(4′-substitutedphenyl)thiazoles with **1** and showed similar NMR characteristics. The characterization data of these compounds are given in Table 1.

However, when 2-hydrazinobenzothiazoles were treated with **1**, only one of the two isomeric products (indicated by ^1H NMR of crude solid) could be obtained having the methyl group at position-3. The C_3-CH_3 protons in all these compounds (**5a-d**) resonated at δ 2.35. The proton decoupled ^{13}C NMR spectrum of **5d** showed that carbon atoms C-3, C-4 and C-5 of the pyrazole moiety res-



Scheme 1

Table 1 – Characterization data of the various compounds prepared

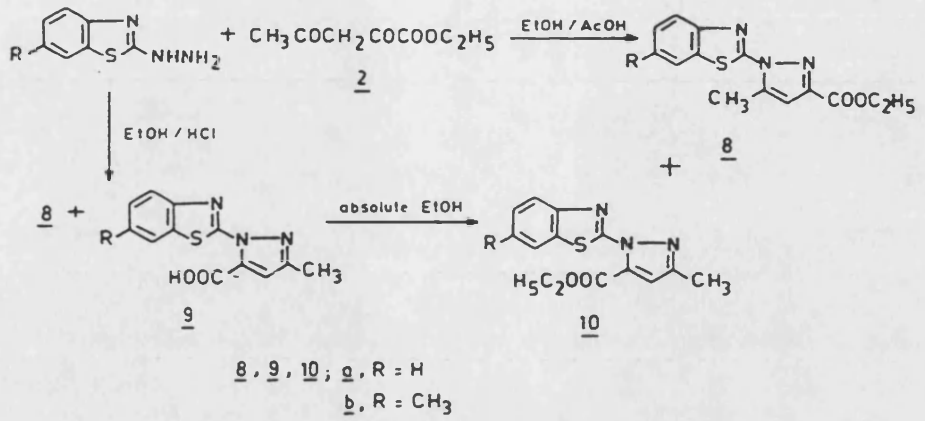
Compd	m.p. °C	Yield (%)	Mol. formula	N (%)	
				Found	Calc.
3a	104	53	$C_{14}H_{10}F_3N_3S$	13.2	13.6
3c	121	38	$C_{14}H_9ClF_3N_3S$	12.0	12.2
4a	115	05	$C_{14}H_{10}F_3N_3S$	13.4	13.6
4c	144	44	$C_{14}H_9ClF_3N_3S$	12.0	12.2
5a	155	71	$C_{12}H_8F_3N_3S$	12.9	13.2
5b	137	74	$C_{13}H_{10}F_3N_3S$	13.8	14.1
5c	138	77	$C_{13}H_{10}F_3N_3OS$	13.1	13.4
8a	110	60	$C_{14}H_{13}N_3O_2S$	14.3	14.6
9a	218(d)	25	$C_{12}H_9N_3O_2S$	16.0	16.2
12a	125	58	$C_{17}H_{17}N_3O_2$	14.3	14.2
13a	216(d)	28	$C_{15}H_{13}N_3O_2$	15.6	15.7
12b	121	56	$C_{17}H_{16}ClN_3O_2$	12.7	12.8
13b	228(d)	25	$C_{15}H_{12}ClN_3O_2$	13.6	14.0

onated at δ 151.69, 112.97 and 133.13 respectively, as observed in the case of **3b**. Complete assignment of other carbon atoms was made on the basis of DEPT technique (*vide* experimental).

These observations reflect the difference in the reactivity of thiazolylhydrazines and benzothiazolylhydrazines. It has already been established that **1** exists in 97% enol form at the $COCF_3$ group in nonpolar solvents[5,6]. Presuming that the pyrazole formation involves nucleophilic attack of the terminal nitrogen on the carbonyl carbon of the β-diketone, it appears likely that the thiazolylhydrazine react rather slowly in comparison to the benzothiazolylhydrazine. However, the present data do not permit us to reach to a definite conclusion on reactivity.

In an attempt to isolate the intermediate of the reaction, 6-chloro-2-hydrazinobenzothiazole was treated with **1** in abs. ethanol at room temperature to give a crystalline solid which appeared to be 5-hydroxy-2-pyrazoline derivative (**6d**) rather than the isomeric hydrazone (**7**). The structure **7** for the intermediate was ruled out as the ^1H NMR spectrum displayed a broad signal (2H) at δ 3.32, a methyl singlet at δ 2.10 and no signal for the olefinic proton around δ 6.0[5]. Formation of **6d** was further supported by recent reports in which similar types of intermediates were isolated[7,8]. As expected, treatment of **6d** in ethanolic HCl gave **5d**.

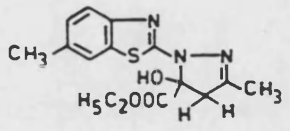
The reaction of hetarylhydrazines with ethyl 2,4-dioxovalerate (**2**) was also investigated. Treatment of 2-hydrazino-6-methylbenzothiazole with **2** in ethanolic HCl afforded a solid whose ^1H NMR spectrum indicated the formation of two products (Scheme 2). Chromatographic separation provided firstly, a compound (**8b**; m.p. 135°) whose ^1H NMR spectrum exhibited two methyl signals at δ 2.45 (C_6-CH_3 of benzothiazole moiety) and 2.81 (C_5-CH_3 of pyrazole moiety) besides signals for ethoxycarbonyl and aromatic protons. The other compound (**9b**; m.p. 245°) exhibited the presence of a carboxyl group in the IR spectrum. The ^1H NMR spectrum revealed it to be a pyrazole derivative with a methyl substituent at position-3 (δ 2.33). The compound could readily be converted into its ethyl ester (**10b**) which was found to be isomeric with **8b** on the basis of ^1H NMR spectral characteristics.



Scheme 2

The structural assignments were further confirmed by an inspection of ^{13}C NMR spectra of **8b** and **9b**. The carbon atoms C-3, C-4 and C-5 of pyrazole moiety in **8b** resonated at δ 145.56, 111.10 and 143.13 and in **9b** at δ 151.59, 112.97 and 136.91, respectively. These values suggest that replacement of methyl by $COOC_2H_5$ or COOH group causes shielding of that carbon by 5-6 ppm[4,9]. Complete assignment of other carbon atoms are given in the experimental section.

However, when the reaction of 2-hydrazino-6-methylbenzothiazole with **2** was carried out in ethanolic acetic acid, a mixture of two isomeric products (**8b** and **10b**) in 3:1 ratio was obtained as indicated by the ^1H NMR spectrum of the crude solid. This observation is in agreement with an earlier report[10] describing the formation of two isomeric products by treatment of **2** with phenylhydrazine in acetic acid. It is thus clear that while using HCl instead of acetic acid, the ethoxycarbonyl group at position-5 gets preferentially hydrolyzed in the reaction medium. Furthermore, treatment of **2** with 2-hydrazino-6-methylbenzothiazole in abs. ethanol produced a mixture of **8b** and 5-hydroxy-2-pyrazoline derivative (**11**) as revealed by ^1H NMR spectrum of the crude solid.



11

Similarly, quinolylhydrazines on condensation with **2** in ethanolic HCl afforded two products having C_5-COOH and $C_3-COOC_2H_5$ groups. This mixture was resolved either by column chromatography or by using sodium bicarbonate. The characterization data of these compounds are given in Table 1.

Experimental Procedure

Melting points are taken in open capillaries and are uncorrected. IR and ^1H NMR spectra were recorded on Beckman IR-20 spectrometer and R-32 Perkin-Elmer (90 MHz) instruments, respectively, ^{13}C NMR spectra on Bruker WH-90 and JEOL GX 270 spectrometers and mass spectra on a Kratos MS-50 instrument at 70 eV.

5-Trifluoromethyl-3-methyl-1-[4′-(4″-tolyl)thiazol-2′-yl]pyrazole (**3b**) *and 3-trifluoromethyl-5-methyl-1-[4′-(4″-tolyl)thiazol-2′-yl]pyrazole* (**4b**)

A mixture of 2-hydrazino-4-(4′-tolyl)thiazole[11] (2.05 g, 0.01 mole) and **1** (1.54 g, 0.01 mole) in ethanol containing a few drops of acetic acid or HCl was refluxed for 3 hr. The reaction mixture was concentrated and left overnight at room temperature. The solid, which separated out indicated the formation of isomeric products **3b** and **4b** in 4:1 ratio as determined by ^1H NMR spectrum. Column chromatographic separation using pet. ether-benzene (60:40) as eluant afforded **3b**, m.p. 124°, yield 62%, ^1H NMR ($CDCl_3$): δ 2.33 (s, 3H, 3-CH_3), 2.36 (s, 3H, 4″-CH_3), 6.64 (s, 1H, H-4), 7.12-7.32 (m, 3H, H-3″, H-5″ and thiazole H-5′), 7.66 (d, 2H, J = 8.3 Hz, H-2″ and H-6″): ^{13}C NMR ($CDCl_3$): δ 13.29 (3-CH_3), 21.14 (4″-CH_3), 109.01 (C-5′), 111.70 (d, $^3J_{C-F}$ = 4 Hz, C-4), 119.52 (q, $^1J_{C-F}$ = 271 Hz, CF_3), 125.78 (C-2″ and C-6″), 129.31 (C-3″ and C-5″), 131.03 (C-1″), 132.20 (q, $^2J_{C-F}$ = 40 Hz, C-5), 138.10 (C-4″), 150.65 (C-3), 152.57 (C-4′), 158.60 (C-2′); MS: m/z 323 (M^+, 100%), 322 (9), 278 (8), 254 (15), 161 (14), 148 (23), 147 (28), 115 (15) (Found: N, 12.9. $C_{15}H_{12}F_3N_3S$ requires N, 13.0%).

Further elution of the column with pet. ether-benzene (60:40) afforded **4b**, m.p. 150°, yield 16%; ^{1}H NMR ($CDCl_3$): δ 2.39 (s, 3H, 4″-CH_3), 2.85 (s, 3H, 5-CH_3), 6.47 (s, 1H, H-4), 7.26 (s, 1H, thiazole H-5′) 7.24 (d, 2H, J = 7.5 Hz, H-3″ and H-5″), 7.77 (d, 2H, J = 8.3 Hz, H-2″ and H-6″); ^{13}C NMR ($CDCl_3$): δ 13.84 (5-CH_3), 21.17 (4″-CH_3), 106.54 (C-4), 109.33 (C-5′), 125.78 (C-2″ and C-6″), 129.38 (C-3″ and C-5″), 131.03 (C-1″), 138.26 (C-4″), 142.67 (C-5), 145.70 (C-3), 152.66 (C-4′), 160.80 (C-2′) (However the CF_3 signal could not be located, perhaps due to the lack of sufficient concentration of the compound); MS: m/z 323 (M^+, 100%), 322 (8), 278 (7), 254 (14), 161 (12), 148 (28), 147 (30), 115 (15) (Found: N, 12.9. $C_{15}H_{12}F_3N_3S$ requires N, 13.0%).

The characterization data of other compounds (**3a**, **3c**, **4a** and **4c**) which were similarly prepared are given in Table 1.

1-(6′-Chlorobenzothiazol-2′-yl)-5-trifluoromethyl-3-methylpyrazole (**5d**)

A mixture of 6-chloro-2-hydrazinobenzothiazole[12] (998 mg, 5 mmoles) and **1** (770 mg, 5 mmoles) in ethanol (25 ml) containing a few drops of conc. HCl was refluxed for 3 hr. The reaction mixture was concentrated and kept at room temperature overnight. The solid thus separated was filtered, dried and crystallised from ethanol, m.p. 170°, yield 75%; ^{1}H NMR ($CDCl_3$): δ 2.35 (s, 3H, 3-CH_3), 6.68 (s, 1H, H-4), 7.31 (dd, 1H, J = 8.8 and 1.8 Hz, H-5′), 7.64 (d, 1H, J = 8.8 Hz, H-4′), 7.69 (d, 1H, J = 1.8 Hz, H-7′); ^{13}C NMR ($CDCl_3$): δ 13.34 (3-CH_3), 112.97 (d, $^3J_{C-F}$ = 3 Hz, C-4), 119.29 (q, $^1J_{C-F}$ = 269 Hz, CF_3), 120.95 (C-7′), 124.11 (C-4′), 127.29 (C-5′) 131.10 (C-6′), 133.13 (q, $^2J_{C-F}$ = 42 Hz, C-5), 134.66 (C-7′a), 149.59 (C-3′a), 151.69 (C-3), 158.70 (C-2′); MS: m/z 317/319 [M^+, 100 (37%)], 282 (4), 249 (4), 248 (22), 221 (8), 169 (9), 133 (9) (Found: N, 12.9. $C_{12}H_7ClF_3N_3S$ requires N, 13.2%).

The characterization data of other members (**5a-c**) prepared similarly are given in Table 1.

1-6′-Chlorobenzothiazol-2′-yl)-5-trifluoromethyl-5-hydroxy-3-methylpyrazole (**6d**)

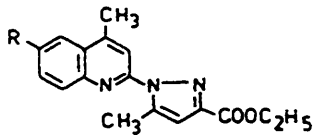
6-Chloro-2-hydrazinobenzothiazole[12] (798 mg, 4 mmoles) and **1** (616 mg, 4 mmoles) were dissolved in ethanol by warming and the solution left overnight at room temperature. The crystalline solid thus obtained was filtered, dried and crystallized from little ethanol, m.p. 115°, yield 85%; IR (nujol): 3250 cm^{-1} (OH); ^{1}H NMR ($CDCl_3$): δ 2.10 (s, 3H, 3-CH_3), 3.32 (bs, 2H, 4-CH_2), 7.28 (dd, 1H, J = 8.2 and 1.8 Hz, H-5′), 7.53 (d, 1H, J = 8.2 Hz, H-4′), 7.65 (d, 1H, J = 1.8 Hz, H-7′).

The other 5-hydroxy-2-pyrazoline derivative (**6c**) was prepared similarly and crystallised from ethanol, m.p. 118°, yield 80%; IR (nujol): 3250 cm^{-1} (OH); ^{1}H NMR ($CDCl_3$): δ 2.16 (s, 3H, 3-CH_3), 3.26 (bs, 2H, 4-CH_2) 3.78 (s, 3H, 6′-OCH_3), 6.87 (dd, 1H, J = 8.8 and 1.8 Hz, H-5′), 7.13 (d, 1H, J = 1.8 Hz, H-7′), 7.46 (d, 1H, J = 8.8 Hz, H-4′).

1-(6′-Methylbenzothiazol-2′-yl)-3-ethoxycarbonyl-5-methylpyrazole (**8b**) *and 1-(6′-methylbenzothiazol-2′-yl)-5-carboxy-3-methylpyrazole* (**9b**) *and its ethyl ester* (**10b**)

A mixture of 2-hydrazino-6-methylbenzothiazole (895 mg, 5 mmoles) and ethyl 2,4-dioxovalerate (**2**, 790 mg, 5 mmoles) in ethanol (30 ml) containing few drops of HCl was refluxed for 2.5 hr. The reaction mixture was concentrated and left at room temperature for 4-5 hr and the separated solid filtered and dried. TLC and ^{1}H NMR spectrum of the crude solid showed the formation of two compounds (3:1). Column chromatographic separation using benzene and then benzene-chloroform (50:50) as eluant afforded **8b** which crystallised from ethanol, m.p. 135°, yield 58%; IR (nujol): 1730 cm^{-1} (C = O); ^{1}H NMR ($CDCl_3$): δ 1.41 (t, 3H, $-COOCH_2CH_3$), 2.45 (s, 3H, 6′-CH_3), 2.81 (s, 3H, 5-CH_3), 4.40 (q, 2H, $-COOCH_2CH_3$), 6.70 (s, 1H, H-4), 7.25 (d, 1H, J = 8.8 and 1.8 Hz, H-5′), 7.61 (d, 1H, J = 1.8 Hz, H-7′), 7.78 (d, 1H, J = 8.8 Hz, H-4′); ^{13}C NMR ($CDCl_3$): δ 13.80 (5-CH_3) 14.12 ($-CH_3$), 21.30 (6′-CH_3), 61.15 ($-OCH_2-$), 111.10 (C-4), 121.09 (C-7′), 122.39 (C-4′), 127.81 (C-5′), 133.39 (C-7′a), 135.36 (C-6′), 143.13 (C-5), 145.56 (C-3), 149.10 (C-3′a), 159.98 (C-2′), 161.59 (CO); MS: m/z 301 (M^+, 100%), 229 (9), 228 (17), 227 (25), 225 (20), 202 (5), 201 (20), 188 (32), 187 (6), 149 (15), 148 (5), 122 (3), 121 (8) (Found: N, 13.7. $C_{15}H_{15}N_3O_2S$ requires N, 14.0%).

Further elution of the column with benzene-chloroform (50:50) afforded **9b** which crystallised from ethanol, m.p. 245°, yield 21%; IR (nujol): 1730 cm^{-1} (C = O); ^{1}H NMR (DMSO-d_6): δ 2.33 (s, 3H, 3-CH_3), 2.50 (s, 3H, 6′-CH_3) 6.85 (s, 1H, H-4), 7.40 (dd, 1H, J = 8.8 and 1.8 Hz, H-5′), 7.83) d, 1H, J = 8.8 Hz, H-4′), 7.89 (bs, 1H, H-7′); ^{13}C NMR (DMSO-d_6): δ 12.94 (3-CH_3), 20.93 (6′-CH_3), 112.97 (C-4), 121.88 (C-7′), 122.01 (C-4′), 128.17 (C-5′), 133.26 (C-7′a), 135.32 (C-6′), 136.91 (C-5), 147.59 (C-3′a), 151.59 (C-3), 158.09 (C-2′), 160.30 (CO); MS: m/z 273 (M^+, 48%), 229 (19), 228 (5), 188 (100), 187 (7), 149 (6), 148 (11), 122(5), 121 (13) (Found: N, 15.3. $C_{13}H_{11}N_3O_2S$ requires N, 15.4%).



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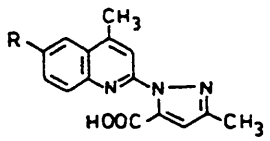

13

12, 13; a, R = H
b, R = Cl

The mixture was also separated by making sodium salt of the acid and then extracting the ester with chloroform. The acid was then regenerated by adding HCl to the sodium salt. The acid **9b** (500 mg) was converted to its ethyl ester (**10b**) by refluxing it in abs. ethanol with a drop of HCl, m.p. 115°, yield 90%; IR (nujol): 1725 cm^{-1} (C=O); ^1H NMR ($CDCl_3$): δ 1.31 (t, 3H, $-COOCH_2CH_3$), 2.36 (s, 3H, 3-CH_3), 2.47 (s, 3H, 6'-CH_3), 4.37 (q, 2H, $-COOCH_2CH_3$), 6.69 (s, 1H, H-4), 7.26 (dd, 1H, J=8.8 and 1.8 Hz, H-5'), 7.64 (bs, 1H, H-7'), 7.79 (d, 1H, J=8.8 Hz, H-4').

When 2-hydrazino-6-methylbenzothiazole and **2** were treated with ethanol containing a few drops of acetic acid, two isomeric esters (**8b** and **10b**) obtained in 3:1 ratio. The characterization data of other compounds (**8a, 9a, 12** and **13**) prepared using 2-hydrazinobenzothiazole and quinolines[13] are given in Table 1.

Acknowledgement

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PUBLICATION 49

Synthesis of Carborane-Containing Nitroimidazole Compounds
via Mild 1,3-Dipolar Cycloaddition

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Synthesis of Carborane-containing Nitroimidazole Compounds *via* Mild 1,3-Dipolar Cycloaddition

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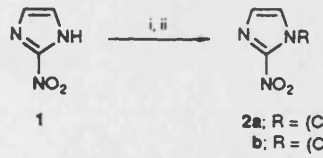
Nitroimidazole-linked carboranes are synthesised in good yield from ω-alkenyl- and ω-alkynyl-2-nitroimidazoles and a carborane nitrile oxide by 1,3-dipolar cycloaddition under mild conditions.

Boron neutron capture therapy (BNCT) is of increasing interest as a strategy for treatment of various cancers[1] and is based on the $^{10}B(n,\alpha)^{7}Li$ reaction of the ^{10}B isotope. Early studies of BNCT using non-targeted boron compounds gave mixed results.[2] Failures were attributed to inadequate concentrations of ^{10}B in the tumour tissue or lack of selectivity of disposition of ^{10}B, leading to damage to normal tissue. Recently, carboranes have been linked to nucleosides[3] and to porphyrins[4] in attempts to target boron to tumours. 2-Nitroimidazoles are selectively retained in poorly vascularised hypoxic tumour tissue by reductive metabolism to electrophiles.[5] As an extension of a programme of synthesis and evaluation of nitroimidazoles in the treatment of cancer,[6,7] we propose that a compound containing 10–12 boron atoms linked to 2-nitroimidazole would form a useful method of concentrating boron in solid tumours. Nitroimidazoles bearing boron are hitherto unreported.

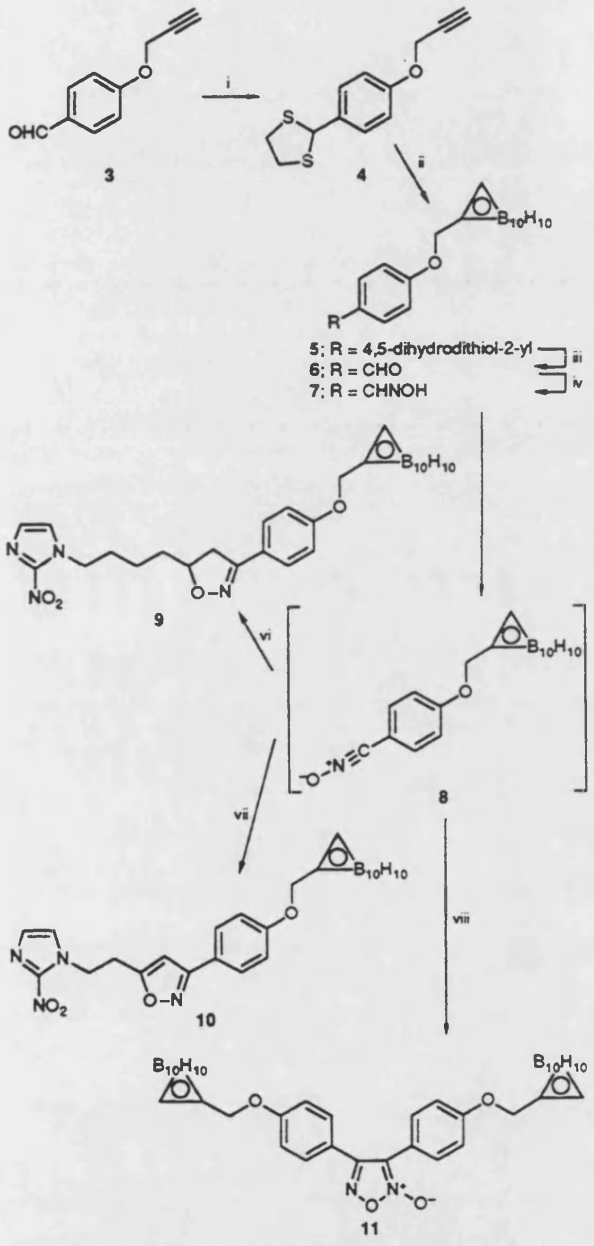
Simple and complex boranes are widely used as reducing agents but 1-substituted-2-nitroimidazoles are themselves readily reduced (E^{1}_{7} = −389 mV);[7] thus assembly of a molecule containing both moieties must be achieved under mild conditions. Alkylation of 2-nitroimidazole **1** requires vigorous conditions and the *closo*-1,2-dicarbadodecarboranes are prepared[8] by reaction of alkynes and decarborane(14) ($B_{10}H_{14}$) in the presence of boiling Lewis bases for long reaction times. 1,3-Dipolar cycloadditions of nitrile oxides to



1

2a; R = $(CH_2)_4CH{=}CH$
b; R = $(CH_2)_2C{\equiv}CH$

Scheme 1 *Reagents and conditions*: i, $KOBu^t$, DMF, 100 °C; ii, $BrCH_2CH_2CH_2CH_2CH{=}CH_2$ or $TsOCH_2CH_2C{\equiv}CH$, DMF, 130 °C



3 4

5; R = 4,5-dihydrodithiol-2-yl
6; R = CHO
7; R = CHNOH

9 8 10 11

Scheme 2 *Reagents and conditions*: i, $HSCH_2CH_2SH$, $BF_3{\cdot}Et_2O$; ii, $B_{10}H_{14}$, MeCN, reflux 3 days; iii, $Hg(ClO_4)_2{\cdot}3H_2O$, THF, 5 min; iv, $NH_2OH{\cdot}HCl$, Na_2CO_3, EtOH; v, NaOCl, H_2O, CH_2Cl_2; vi, **2a**; vii, **2b**; viii, PhMe, reflux

alkenes and alkynes proceed[9] under mild conditions; hence this method was chosen to link appropriate nitroimidazoles and carboranes for the final assembly step.

The potassium salt of 2-nitroimidazole **1** was alkylated with 6-bromohex-1-ene and with but-3-ynyl tosylate[10] in hot DMF (dimethylformamide) to give the alkene **2a**[7] (78%) and the alkyne **2b**† (59%), respectively (Scheme 1). As predicted, treatment of **2b** with $B_{10}H_{14}$ gave only polar degradation products.

4-(3-Prop-1-ynyloxy)benzaldehyde **3**[11] was chosen as the bifunctional compound for elaboration to form a carborane and a nitrile oxide. Both aldehyde **3** and the 4,5-dihydro-1,3-dioxole protected form were unstable to $B_{10}H_{14}$. However, protection of the aldehyde as the 4,5-dihydro-1,3-dithiole **4**† was achieved in 80% yield (Scheme 2). This protecting group resisted prolonged treatment with $B_{10}H_{14}$ in refluxing acetonitrile, which furnished carborane **5**† (51%). The aldehyde was unmasked cleanly under very mild conditions using $Hg(ClO_4)_2$, giving carboranylmethoxybenzaldehyde **6**† (90%) in a much shorter sequence than that reported[4] for the synthesis of the *meta* isomer. Oxidation of the corresponding oxime **7**† to give nitrile oxide **8**‡ and 1,3-dipolar cycloaddition with **2a** and **2b** were effected§ as one-pot procedures, affording the required dihydroisoxazole **9**†¶ and the isoxazole **10**†‖ in which both nitroimidazole and carborane moieties are present. Yields were essentially quantitative based on dipolarophile and nitrile oxide consumed.

Formation of the intermediate nitrile oxide **8** was very rapid but prolonged reaction times at ambient temperature were required for acceptable conversion into heterocycles. Even after several days, no boron-containing compounds other than **8**, **9** or **10** were evident and it was possible to isolate unreacted **8** from the reaction mixtures by chromatography. This nitrile oxide is remarkably stable, with little decomposition after several weeks at ambient temperature; in contrast, the $t_{\frac{1}{2}}$ of most aromatic nitrile oxides is reported[9] to be only a few hours. Conversion into the dimer, 1,2,5-oxadiazole 2-oxide **11**,†** was effected only on heating in boiling toluene.

The mild conditions of the 1,3-dipolar cycloaddition described here permit the joining of sensitive 2-nitroimidazole and boron cage moieties within one molecule. This strategy represents an opportunity for incorporating chemically sensitive pharmacophores and targeting groups into drug molecules while generating a heterocycle which is itself capable of further elaboration. Compounds **9** and **10** are highly

† New compounds were characterised by ^1H NMR and MS and, for target compounds, microanalysis or high resolution MS.

‡ *Spectroscopic data* **8**: IR ν_{max}/cm^{-1} 2600 (B–H) and 2320 ($C{\equiv}N^+{-}O^-$); NMR ($CDCl_3$) δ 1.2–3.1 (10 H, br m, B_{10}-H_{10}), 3.97 (1 H, s, carborane 2-H), 4.38 (2 H, s, carborane-CH_2), 6.82 (2 H, d, Ar 3,5-H_2) and 7.40 (2 H, d, Ar 2,6-H_2).

§ Typical experiment: oxime **7** (1 mmol) and alkyne **2b** (1 mmol) in CH_2Cl_2 (20 ml) were treated with aqueous NaOCl for 18 h. Chromatography (silica gel; CH_2Cl_2) of the evaporation residue gave isoxazole **10**.

¶ **9**: NMR ($CDCl_3$) δ 1.3–3.3 (10 H, br m, B_{10}-H_{10}), 1.5–2.0 (6 H, m, imidazole-$CH_2CH_2CH_2CH_2$), 2.93 (1 H, dd) and 3.40 (1 H, dd) isoxazole 4-H_2, 4.09 (1 H, br, carborane 2-H), 4.45 (4 H, m, imidazole-CH_2 + carborane-CH_2), 4.73 (1 H, ddt, isoxazole 5-H), 6.87 (2 H, d, Ar 3,5-H_2) 7.11 (1 H, s) and 7.15 (1 H, s) imidazole 4,5-H_2, and 7.61 (2 H, d, Ar 2,6-H_2).

‖ **10**: NMR ($CDCl_3$) δ 1.3–3.3 (10 H, br m, B_{10}-H_{10}), 3.42 (2 H, t, isoxazole-CH_2), 4.09 (1 H, br, carborane 2-H), 4.46 (2 H, s, carborane-CH_2), 4.82 (2 H, t, imidazole-CH_2), 6.26 (1 H, s, isoxazole 4-H), 6.92 (2 H, d, Ar 3,5-H_2), 6.96 (1 H, s) and 7.09 (1 H, s) imidazole 4,5-H_2, and 7.70 (2 H, d, Ar 2,6-H_2).

** **11**: NMR ($CDCl_3$) δ 1.3–3.3 (20 H, br, m, 2 × B_{10}-H_{10}), 4.07 (2 H, br, 2 × carborane 2-H), 4.46 (2 H, s, carborane-CH_2), 4.47 (2 H, s, carborane-CH_2), 6.92 (4 H, d, Ar 3,5-H_2 + Ar' 3,5-H_2), 7.47 (2 H, d, Ar 2,6-H_2), and 7.49 (2 H, d, Ar' 2,6-H_2).

lipophilic; the development of more water-soluble analogues for biological evaluation will be reported elsewhere.

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Synthesis and Biological Evaluation of a Series of Flavones Designed as Inhibitors of Protein Tyrosine Kinases

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Synthesis and biological evaluation of a series of flavones designed as inhibitors of protein tyrosine kinases

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Summary: A series of flavones has been prepared, which are variously substituted in the 3,3',4',5 and 7 positions with halo-, alkoxy-, nitro-, amino-, hydroxy-, acyloxy- and azido-groups, for evaluation of their cytotoxicity to ANN-1 cells (3T3 murine fibroblasts transformed with the Abelson murine leukaemia virus) which contain a tyrosine kinase. This cytotoxicity was compared to their non-transformed 3T3 counterparts. 3'-Amino-4'-methoxyflavone was the most cytotoxic compound (IC_{50} = 1.6 μM) and was less inhibitory to the non-transformed parent 3T3 cell line (IC_{50} = 8 μM). The compound was inactive at 50 μM in assays of the inhibition of the cell-associated Abelson protein tyrosine kinase but inhibited an epidermal growth factor (EGF) protein tyrosine kinase by 42% at 50 μM. Quercetin (3,3',4',5,7-pentahydroxyflavone) was the most potent inhibitor of the Abelson protein tyrosine kinase but showed no selective inhibition of the growth of ANN-1 cells compared to the parent 3T3 cell line. Different structure–activity relationships were observed between the results of the cytotoxicity assays and inhibition of protein tyrosine kinases. Inhibitors of the Abelson protein tyrosine kinase which were competitive with respect to ATP showed different potencies for inhibition of the EGF receptor kinase.

Key words: Abelson kinase/ANN-1 cells/flavone/protein tyrosine kinase/synthesis

Introduction

In the search for new and more selective agents for the treatment of cancer, the modulation of biochemistry associated with the cell membrane, which acts to undermine the normal regulation of proliferation and differentiation (Heldin *et al.*, 1987), presents itself as an attractive pharmacological strategy (Tritton & Hickman, 1990; Powis, 1991). It is at this cellular locus that the products of oncogenes are expressed in such a way as to subvert growth regulatory processes. The protein tyrosine kinases encoded by oncogenes (Yarden & Ullrich, 1988) have become targets for the medicinal chemist.

Several natural and synthetic compounds inhibit these kinases, including the isoflavone genistein (1; Chart I) (Akiyama *et al.*, 1987), the diuretic amiloride (2) (Davis & Czech, 1985) and the alkaloid staurosporine (3) (Nakano *et al.*, 1987). The disadvantage

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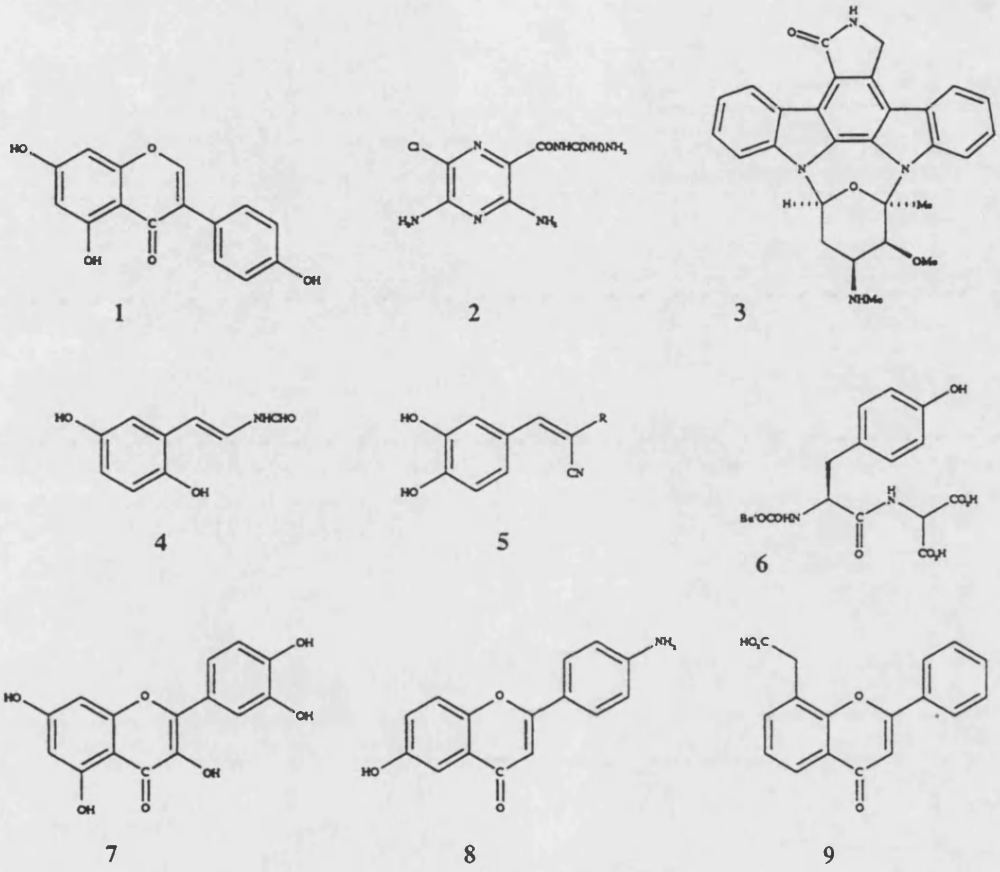


Chart I Structures of known inhibitors of protein tyrosine kinases and analogous flavones; **1**: genistein; **2**: amiloride; **3**: staurosporine; **4**: erbstatin; **5**: tyrphostins; **6**: BOCtyrosylaminomalonic acid; **7**: quercetin; **8**: 4′-amino-6-hydroxyflavone; **9**: flavone-8-acetic acid

common to all these compounds is their poor selectivity towards inhibition of purified tyrosine kinases when compared to inhibition of the serine and threonine kinases. Synthetic multisubstrate analogues, incorporating features of the $[ATP\cdots Tyr]^{\ddagger}$ transition state, inhibit both the transforming Abelson viral tyrosine kinase $p60^{v\text{-}abl}$ and the Ser/Thr specific phosphorylase kinase (Kruse *et al.*, 1988). Erbstatin (**4**) and synthetic benzylidenemalononitrile analogues have been described as selective competitive substrate inhibitors of the epidermal growth factor (EGF) receptor tyrosine kinase (Yaish *et al.*, 1988). The activities of these 'tyrphostins' (**5**) were demonstrated (Gazit *et al.*, 1987) to show $IC_{50} < 100$ μM as competitive reversible inhibitors of the insulin receptor tyrosine kinase and were minimally cytotoxic at 100 μM towards A431 cells, but were cytostatic. A recent study has, however, suggested that their rôle in growth inhibition may not only involve inhibition of cellular EGF receptor tyrosine kinase activity (Faaland *et al.*, 1991). BOCtyrosylaminomalonic acid (**6**) has been reported to be a

competitive inhibitor of the tyrosine kinase activity of the insulin receptor (Schechter *et al.*, 1989), but phosphonic acid analogues of phosphotyrosine are largely inactive (Burke *et al.*, 1991).

For our study, quercetin (7) was chosen as a lead compound. This flavone inhibits the tyrosine kinase activity of the Rous sarcoma viral kinase $pp60^{v\text{-}src}$ (Graziani *et al.*, 1983). The selectivity of the flavones and isoflavones is notable since quercetin is a competitive inhibitor at the ATP binding site of many enzymes, a region of considerable homology amongst kinases (Hanks *et al.*, 1988). The polyhydroxylated flavones have been described as having a promiscuous pharmacology (Havsteen, 1983), although considerable specificity can be achieved against kinases. Cushman *et al.* (1991) disclosed recently that a wide range of flavone-3-carboxylates are inactive against the protein tyrosine kinase activity of $p56^{lck}$, whereas potency 10-fold greater than that of quercetin was shown by 4'-amino-6-hydroxyflavone (**8**).

One of our goals was to synthesize flavones which have greater selectivity when compared with quercetin whilst retaining or increasing potency. The effects of these novel compounds on the proliferation of normal 3T3 murine fibroblasts were compared with the effects on ANN-1 cells (3T3 fibroblasts transformed by the Abelson murine leukaemia virus) (Scher & Siegler, 1975). Such a comparison would facilitate the identification of those compounds exerting their effects specifically through inhibition of the Abelson kinase from those exhibiting a non-selective cytotoxicity. Selected compounds were assayed against the tyrosine kinase activities of either a recombinant protein, p*tabl*50, from the Abelson virus or on extracts from the ANN-1 cells. Additionally, the effects of the most active of our compounds on the activities of the EGF receptor protein tyrosine kinase and of a cyclic AMP-dependent protein kinase were assayed to assess selectivity.

Methods

Synthetic chemistry

Benzoate esters were synthesized by Methods A, B and C, propane-2,4-diones were synthesized by Methods D and E, and flavones were synthesized by Methods F–O, below. NMR spectra at 60, 300 and 400 MHz were obtained using Varian EM360A, Bruker AC300 and Bruker WH400 spectrometers, respectively. Electron-impact mass spectra were obtained using VG Micromass 12B and ZAB-E instruments. Compounds **11, 12, 30, 33, 35, 37, 43–45, 49, 52, 54, 56** and **62–64** were prepared as previously described (Cunningham *et al.*, 1989).

Method A. The substituted benzoyl chloride (35 mmol) was stirred with the substituted 2-acetylphenol (25 mmol) in pyridine (20 ml) for 30 min. The mixture was added to an excess of hydrochloric acid (1 M) at 0°C. The solid was recrystallized from methanol to give the benzoate ester.

Method B. The substituted benzoyl chloride (40 mmol) was stirred with the substituted 2-acetylphenol (30 mmol) and 4-(dimethylamino)pyridine (3 mmol) in pyridine (15 ml) for 10 min. The ester was isolated as for Method A.

Method C. The substituted benzoyl chloride (60 mmol) was stirred with the substituted 2-acetylphenol (25 mmol) in pyridine (20 ml) for 30 min. The ester was isolated as for Method A.

Method D. Powdered potassium hydroxide (30 mmol) was added to the substituted benzoate ester (20 mmol) in pyridine (50 ml) at 50°C. The mixture was stirred at 50°C for 20 min, then added to an excess of hydrochloric acid (1 M). The solid was recrystallized from methanol to give the substituted propane-1,3-dione.

Method E. Butyl lithium (20 mmol; 1.5 M in hexanes) was added to hexamethyldisilazane (20 mmol) in dry tetrahydrofuran (50 ml) at −78°C under N_2 and the mixture was stirred for 30 min. The benzoate ester (10 mmol) in dry tetrahydrofuran (20 ml) was added during 15 min at −78°C. The mixture was warmed to 20°C during 2 h and an excess of hydrochloric acid (1 M) was added. Recrystallization of the solid (methanol) gave the substituted propane-1,3-dione.

Method F. The substituted propane-1,3-dione (10 mmol) was boiled under reflux with concentrated sulphuric acid (1 ml) in acetic acid (30 ml) for 1 h. The mixture was added to ice. The solid was recrystallized to give the substituted flavone.

Method G. Boron tribromide (6 mmol) was stirred with the methoxyflavone (2 mmol) in dichloromethane (50 ml) for 16 h. The reaction was quenched with aqueous sodium hydroxide (10%), acidified with hydrochloric acid (9 M) and extracted with dichloromethane. The organic solvent was evaporated to a small volume and the hydroxyflavone was collected.

Method H. The methoxyflavone (2 mmol) was boiled under reflux for 7 days with hydrogen bromide in acetic acid (15% w/w; 20 ml). The cooled mixture was quenched with aqueous sodium hydroxide (10%), acidifed with hydrochloric acid (1 M) and extracted with dichloromethane. Recrystallization of the evaporation residue gave the hydroxyflavone.

Method I. The chloroflavone (2.5 mmol) was boiled under reflux with aqueous dimethylamine (40% w/w; 50 ml) for 2 h. The mixture was poured onto ice and hydrochloric acid. Neutralization with aqueous sodium hydroxide gave a solid which was recrystallized to give the dimethylaminoflavone.

Method J. The nitroflavone (1.5 mmol) was boiled under reflux with tin (II) chloride (7.5 mmol) in hydrochloric acid (9 M; 20 ml) for 1 h, then cooled to 0°C. Recrystallization of the solid gave the aminoflavone.

Method K. The nitroflavone (0.5 mmol) was boiled under reflux with tin (II) chloride (7.5 mmol) and hydrochloric acid (9 M; 1 ml) in ethanol (20 ml) for 1 h. The cooled mixture was basified with excess aqueous sodium hydroxide (10 M) and was extracted with dichloromethane. Drying (anhydrous sodium sulphate) and evaporation of the solvent under reduced pressure gave the diaminoflavone as an oil which darkened on exposure to air.

Method L. Sodium nitrite (2.7 mmol) in water (15 ml) was added during 15 min to the aminoflavone (2.5 mmol) in hydrochloric acid (5 M; 5 ml) at 0°C. After a further 15 min at 0°C, sodium azide (10 mmol) (CAUTION!) was added during 30 min. After a further 30 min, the mixture was poured onto ice and was basified with aqueous ammonia. The solid was washed with water and recrystallized to give the azidoflavone.

Method M. The flavone ester (2 mmol) was stirred with aqueous potassium hydroxide

(20%; 20 ml) for 1 h at 60°C, then poured onto an excess of hydrochloric acid (1 M) at 0°C. The solid was suspended in aqueous sodium hydrogen carbonate for 30 min at 50°C, then collected. Recrystallization gave the hydroxyflavone.

Method N. Butyl lithium (1.6 M in hexanes; 6.25 ml) was added to diisopropylamine (10 mmol) in dry tetrahydrofuran (20 ml) at −78°C, followed, after 15 min, by the flavone (10 mmol). After a further 15 min at −78°C, trimethyl borate (10 mmol) was added and the mixture was stirred for 30 min. Acetic acid (15 mmol) and hydrogen peroxide (30% in water; 1.2 ml) were then added during 15 min. The mixture was extracted with ethyl acetate and the extract was washed with aqueous sodium hydrogen carbonate. The evaporation residue was recrystallized to give the 3-hydroxyflavone.

Method O. Sulphuryl chloride (11 mmol) was stirred with flavone (10 mmol) in tetrachloromethane (30 ml) for 24 h. Chromatography (silica gel; chloroform) gave the 3-chloroflavone and the trichlorodihydroflavone.

Cytotoxicity assays and biochemical methods

Cytotoxicity assays. 3T3 cells and ANN-1 cells (Scher & Siegler, 1975), kind gifts of Dr Gordon Foulkes, were grown in Dulbecco's modified Eagles medium, supplemented with 10% fetal calf serum. Both had a doubling time of 18 h. ANN-1 cells grew in suspension culture, whereas the 3T3 cell line grew as a monolayer. Cells (2×10^4) were treated with a range of concentrations of test compounds. Compounds were dissolved in dimethylformamide (DMF) or dimethylsulphoxide (DMSO) such that the final concentrations of these solvents did not exceed 0.5% v/v. The cells were incubated for 3 days in the presence of each agent prior to assay of cell numbers. Following aspiration to disperse clumps, ANN-1 cells were counted using a Coulter Counter model ZM. 3T3 cells were washed with Ca^{2+}-free and Mg^{2+}-free phosphate-buffered saline solution and were treated with 0.2% trypsin in saline solution until the cells detached from the substratum. Fresh medium (1 ml) was added and the cells were aspirated to disperse clumps prior to counting. The inhibition of cell growth was calculated as the percentage change in the increase in cell number in the treated cells compared to that of the controls which had received DMF or DMSO alone.

Assays of protein tyrosine kinase activity. The activity of the Abelson protein tyrosine kinase was assayed by the use of either a purified, cloned fragment of the kinase (pt*abl*50) (a kind gift of Dr Gordon Foulkes) or by the use of a cellular lysate from ANN-1 cells. For assays with the purified enzyme, a sample (10 μl) of the pt*abl*50 kinase (0.165 mg/ml) was diluted 1:100 with enzyme buffer containing EDTA (0.1 mM), Brij 35 (0.05%), dithiothreitol (1.0 mM) and bovine serum albumin (BSA) (0.1 mg/ml) in PIPES buffer (50 mM) at pH 7.5. To this were added 10 μl of a solution of angiotensin I (3 mg/ml) in the assay buffer which contained EDTA (0.1 mM) and Brij 35 (0.05%) in PIPES (50 mM) buffer at pH 7.5. The solution of the test compound or solvent (5 μl) was added (see above) and the reaction was initiated by the addition of 0.3 mM γ-[^{32}P]ATP (specific activity 600–1000 c.p.m./pmol; 10 μl). The mixture was incubated at 30°C for 30 min before being quenched with aqueous phosphoric acid (10% v/v; 100 μl). A portion of the reaction mixture (100 μl) was spotted onto Whatman p81 phosphocellulose paper which was washed extensively with 6% aqueous acetic acid and once with acetone, and dried. The radioactivity was counted using a Packard Tricarb CA2000 scintillation counter.

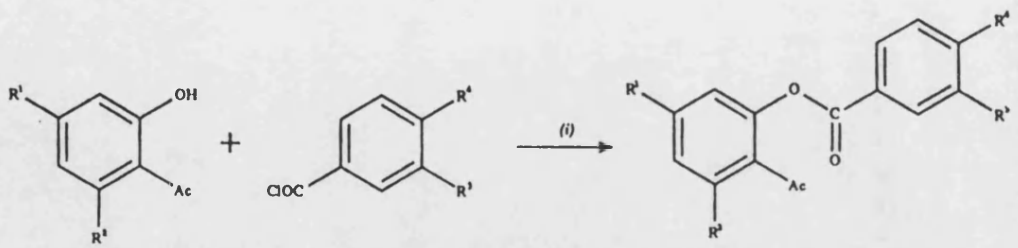
When the cell lysate was used, 10^7 ANN-1 cells were lysed for 10 min at 37°C in a

buffer containing EDTA (0.2 mM), sodium vanadate (100 μM), Triton X-100 (1%) and dithiothreitol (1.0 mM) in PIPES buffer (50 mM) at pH 7.5. The suspension was centrifuged at 600 *g* for 5 min. The supernatant was diluted 1:10 with enzyme buffer and was used as for p*tabl*50 above, except that the reaction was quenched with 5% aqueous trichloroacetic acid. For assays of EGF receptor tyrosine kinase activity, membranes were prepared from 10^8 A431 cells/ml by the method of Pike (1987) and were stored at −20°C in a buffer comprising NaCl (100 mM) and NP-40 detergent (0.1%) in HEPES buffer (20 mM) at pH 7.5. The same buffer was used to prepare stock solutions of EGF (0.4 μM), RR-SRC peptide (Casnellie *et al.*, 1982) (4.0 mM) and an ATP cocktail of γ-[^{32}P]ATP (400 μM; 125 nCi/ml), supplemented with $MnCl_2$ (16 mM), $Zn(OAc)_2$ (40 μM) and sodium vanadate (400 μM). The A431 membrane preparation (4 μl), the EGF stock solution (4 μl), the solution of RR-SRC peptide (4 μl) and the test compound or solvent (DMF or DMSO) were preincubated for 5 min before the reaction was initiated by addition of the ATP solution (4 μl). Incubation continued for a further 5 min. Non-specific phosphorylation was assayed in incubations in the absence of either the EGF or the RR-SRC peptide. The reaction was stopped by adding 3.5% aqueous trichloroacetic acid (184 μl) at 0°C. The mixture was centrifuged at 13 500 *g* for 2 min. A sample (100 μl) of the supernatant was spotted onto Whatman p81 phosphocellulose paper and was washed with aqueous phosphoric acid (75 mM; 5 ml). The content of ^{32}P was measured as above. Phosphorylation of the RR-SRC peptide by the crude membrane preparation was found to be linear with time. Phosphorylation by unstimulated membranes remained <15% of the levels observed during stimulation.

Chemistry

A series of flavones variously substituted in the 3,3′,4′,5 and 7 positions was sought, these positions corresponding to the positions of the potentially hydrogen-bonding hydroxy groups in quercetin (**7**). These flavones were generally more hydrophobic than **7**. The method of Baker (1933), involving acid-catalysed cyclodehydration of substituted 1-(2-hydroxyphenyl)-3-phenylpropane-1,3-diones, has been the most widely employed and was selected for the synthesis of many of the flavones (2-phenylbenzo-4*H*-pyran-4-ones) in this study. 2-Acetylphenol (**10**) was esterified by benzoyl chloride (**14**). Baker–Venkataraman rearrangement of the resulting ester (**29**) was effected using powdered potassium hydroxide in pyridine. The final cyclodehydration was carried out with catalytic sulphuric acid in hot acetic acid to give flavone (**66**). In some cases, the base-catalysed rearrangement did not proceed satisfactorily with $^-$OH, but *O* to *C* migration of the acyl group in esters **42–44** using lithium hexamethyldisilazide as base enabled the synthesis of the propane-1,3-diones **61–63**. Flavones **66–79**, bearing 3′ and/or 4′ substituents which are stable to the various sets of reaction conditions, were easily approached using suitably substituted benzoyl chlorides **15–27** through esters **29–41** and through propane-1,3-diones **48–60** (Scheme I). Yields, analyses and spectroscopic data are given in Tables I–IV.

The introduction of the more labile 3′ and 4′ substituents was carried out using several methods. Flavone **86** substituted with a hydroxy group at position 3′ was prepared by demethylation of the corresponding methyl ether using hydrogen bromide in acetic acid (Scheme II). The rate of demethylation was considerably enhanced by the use of boron tribromide, enabling the relatively rapid formation of 3′,4′-dihydroxyflavone (**85**) and hydroxynitroflavones **87** and **88** from methoxy precursors **68**, **77** and **78**. Treatment of 4′-chloro-3′-nitroflavone (**76**) with boiling aqueous dimethylamine furnished the dimethylamino compound **89** (Scheme III). The (primary amino)flavones **90–94** were generated efficiently by the reduction of nitroflavones **71**, **72**, **76**, **77** and **78** with tin (II) chloride



10: R^1, R^3 = H
11: R^1, R^3 = QMe
12: R^1 = OMe; R^3 = H
13: R^1 = OH; R^3 = H

14: R^3, R^4 = H
15: R^3 = P; R^4 = H
16: R^3, R^4 = OMe
17: R^3 = H; R^4 = OMe
18: R^3 = OMe; R^4 = H
19: R^3 = H; R^4 = NO_2
20: R^3 = NO_2; R^4 = H

21: R^3 = H; R^4 = Cl
22: R^3 = H; R^4 = Br
23: R^3, R^4 = Cl
24: R^3 = NO_2; R^4 = Cl
25: R^3 = NO_2; R^4 = OMe
26: R^3 = OMe; R^4 = NO_2
27: R^3 = Cl; R^4 = H

(i)

28: R^2, R^3, R^5, R^4 = H
29: R^2, R^5 = H; R^3 = P; R^4 = H
30: R^2, R^5 = H; R^3, R^4 = OMe
31: R^2, R^5, R^3 = H; R^4 = OMe
32: R^2, R^5 = H; R^3 = OMe; R^4 = H
33: R^2, R^5, R^3 = H; R^4 = NO_2
34: R^2, R^5 = H; R^3 = NO_2; R^4 = H
35: R^2, R^5, R^3 = H; R^4 = Cl
36: R^2, R^5 = H; R^3 = Br
37: R^2, R^5 = H; R^3, R^4 = Cl

38: R^1, R^3 = H; R^2 = NO_2; R^4 = Cl
39: R^1, R^3 = H; R^2 = NO_2; R^4 = OMe
40: R^1, R^3 = H; R^2 = OMe; R^4 = NO_2
41: R^1, R^3 = H; R^2 = Cl; R^4 = H
42: R^1 = OMe; R^3, R^5 = H; R^4 = NO_2
43: R^1, R^3 = OMe; R^5, R^4 = H
44: R^1 = OMe; R^3, R^5, R^4 = H
45: R^1 = O_2CPh; R^3, R^5, R^4 =H
46: R^1 = *m*-$O_2CC_6H_4OMe$; R^3 = H; R^2 = OMe; R^4 = H

(ii) or (iii)

47: R^2, R^5, R^3, R^4 = H
48: R^2, R^5 = H; R^3 = P; R^4 = H
49: R^2, R^5 = H; R^3, R^4 = OMe
50: R^2, R^5, R^3 = H; R^4 = OMe
51: R^2, R^5 = H; R^3 = OMe; R^4 = H
52: R^2, R^5, R^3 = H; R^4 = NO_2
53: R^2, R^5 = H; R^3 = NO_2; R^4 = H
54: R^2, R^5, R^3 = H; R^4 = Cl
55: R^2, R^5, R^3 = H; R^4 = Br
56: R^2, R^5 = H; R^3, R^4 = Cl

57: R^1, R^3 = H; R^2 = NO_2; R^4 = Cl
58: R^1, R^3 = H; R^2 = NO_2; R^4 = OMe
59: R^1, R^3 = H; R^2 = OMe; R^4 = NO_2
60: R^1, R^3 = H; R^2 = Cl; R^4 = H
61: R^1 = OMe; R^2, R^5 = H; R^4 = NO_2
62: R^1, R^3 = OMe; R^5, R^4 = H
63: R^1 = OMe; R^2, R^5, R^4 = H
64: R^1 = O_2CPh; R^3, R^5, R^4 =H
65: R^1 = *m*-$O_2CC_6H_4OMe$; R^3 = H; R^2 = OMe; R^4 = H

(iv)

66: R^2, R^5, R^3, R^4 = H
67: R^2, R^5 = H; R^3 = P; R^4 = H
68: R^2, R^5 = H; R^3, R^4 = OMe
69: R^2, R^5, R^3 = H; R^4 = OMe
70: R^2, R^5 = H; R^3 = OMe; R^4 = H
71: R^2, R^5, R^3 = H; R^4 = NO_2
72: R^2, R^5 = H; R^3 = NO_2; R^4 = H
73: R^2, R^5, R^3 = H; R^4 = Cl
74: R^2, R^5, R^3 = H; R^4 = Br
75: R^2, R^5 = H; R^3, R^4 = Cl

76: R^1, R^3 = H; R^2 = NO_2; R^4 = Cl
77: R^1, R^3 = H; R^2 = NO_2; R^4 = OMe
78: R^1, R^3 = H; R^2 = OMe; R^4 = NO_2
79: R^1, R^3 = H; R^2 = Cl; R^4 = H
80: R^1 = OMe; R^2, R^3 = H; R^4 = NO_2
81: R^1, R^3 = OMe; R^2, R^4 = H
82: R^1 = OMe; R^2, R^3, R^4 = H
83: R^1 = O_2CPh; R^2, R^3, R^4 =H
84: R^1 = *m*-$O_2CC_6H_4OMe$; R^2 = H; R^3 = OMe; R^4 = H

Scheme I Synthesis of flavones by cyclization of substituted propane-1,3-diones. Reagents: (i) pyridine; (ii) KOH/pyridine; (iii) $LiN(SiMe_3)_2$/tetrahydrofuran; (iv) H_2SO_4/HOAc

Table I Yields and properties of benzoate esters

Benzoyl chloride	*2-Acetylphenol*	*Benzoate ester*	*Method*	*Yield (%)*	*m.p. (°C)*	*Elemental analysis*			
						C (%)	*H (%)*	*N (%)*	
14	**10**	**28**	A	78	89–90[a]				
15	**10**	**29**	A	74	65	69.76	4.29		calc'd
						69.58	4.33		found
17	**10**	**31**	A	85	116[b]				
18	**10**	**32**	A	53	52	71.10	5.22		calc'd
						71.11	5.22		found
20	**10**	**34**	A	75	94[c]				
22	**10**	**36**	A	73	94[d]				
24[e]	**10**	**38**	A	55	99–100	56.35	3.15		calc'd
						56.36	3.16		found
25[f]	**10**	**39**	A	86	139–140	60.95	4.15	4.44	calc'd
						60.83	4.20	4.46	found
26[g]	**10**	**40**	A	86	108–109	60.95	4.15	4.44	calc'd
						60.78	4.13	4.49	found
27	**10**	**41**	A	81	84	65.58	4.03		calc'd
						65.68	4.05		found
19	**12**	**42**	B	68	141[h]				
18	**13**	**46**	C	81	67–68[i]	67.79	5.00		calc'd[i]
						67.68	5.00		found[i]

[a] lit. (Furniss *et al.* 1981) m.p. 87–88°C
[b] lit. (Baker & Glockling, 1950) m.p. 113–114°C
[c] lit. (Virkar, 1942) m.p. 99–100°C
[d] lit. (Baker *et al.*, 1952) m.p. 92–93°C
[e] Al-Jallo & Jalhoom (1972)
[f] Wu & Herbst (1952)
[g] Liss (1952)
[h] lit. (Wadodkar & Marathey, 1972) m.p. 135–137°C
[i] Mono-methanol solvate

(Scheme IV). Reduction of the nitro function of **89** gave the diamine **95** which autoxidized too rapidly to permit meaningful biological evaluation. Diazotization of amines **90** and **91**, and subsequent treatment with sodium azide, gave the azidoflavones **96** and **97** in good yields.

Preparation of flavones substituted at positions 7 and 5 required ring-substituted phenols. Attempted Fries rearrangement of 3-nitrophenyl acetate to 2-acetyl-4-nitrophenol with aluminium trichloride (leading towards 7-aminoflavones) failed owing to deactivation of the benzene ring. As reported previously (Cunningham *et al.*, 1989), Lewis acids caused degradation of the electron-rich mono- and di-methoxyphenyl acetates, hence the direct acetylation of 3-methoxyphenol and 3,5-dimethoxyphenol *ortho* to the hydroxy group was effected using boron trichloride generally by the method of Piccolo *et al.* (1986). Introduction of hydroxy groups at position 7 of flavones was achieved through the diacylation of 1-acetylbenzene-2,4-diol (**13**) by appropriate benzoyl chlorides **14** and **18**, giving the diesters **45** and **46** (Scheme I). The esters of propane-1,3-diones **64** and **65** were stable to the conditions of base-catalysed rearrangement (potassium hydroxide in hot pyridine), probably owing to the exclusive presence of the β-ketoenolates. Cyclization to the acyloxyflavones **83** and **84** was effected under the

Table II Yields and properties of propane-1,3-diones

Benzoate ester	*Propane-1,3-dione*	*Method*	*Yield (%)*	*m.p. (°C)*	*Elemental analysis*		
					C (%)	*H (%)*	
28	**47**	D	63	124[a]			
31	**50**	D	66	111[b]			
42	**51**	D	70	84–88	71.10	5.22	calc'd
					70.74	5.26	found
34	**53**	D	72	150[c]			
36	**55**	D	42	142	56.45	3.47	calc'd
					56.75	3.54	found
38	**57**	D	54	196[d]			
39	**58**	D	95	171	60.95	4.15	calc'd
					61.09	4.28	found
40	**59**	D	54	188–189			calc'd
					60.95	4.15	found
					60.55	4.21	
41	**60**	D	56	117–118[e]			
42	**61**	E	48	200	60.95	4.15	calc'd
					60.71	4.13	found

[a] lit. (Furniss *et al.*, 1981) m.p. 121°C
[b] lit. (Wheeler, 1963) m.p. 111°C
[c] lit. (Virkar, 1942) m.p. 157°C
[d] lit. (Jucker & Vogel, 1963) m.p. 189–190°C
[e] lit. (Netherlands Patent, 1965) m.p. 115°C

usual acid-catalysed conditions. Subsequent basic hydrolysis of **84** furnished the 7-hydroxy-3'-methoxyflavone (**98**) (Scheme V).

Functionalization of flavones at the 3-position was investigated. Costa *et al.* (1985) reported lithiation of the 3-position of flavone (**66**) with lithium diisopropylamide. Treatment of this anion with trimethyl borate, followed by oxidation with hydrogen peroxide, gave the required 3-hydroxyflavone (**99**) (Scheme VI). The general applicability of this method is limited by the acidity of the 3-proton. Anions were not formed from methoxyflavones **69** and **70**, as shown by quench either with trimethyl borate or with deuteroacetic acid. The corresponding nitroflavones were very poorly soluble in solvents appropriate for lithiation reactions. Since a chlorine atom is approximately isosteric with a hydroxy group, the synthesis of 3-chloroflavone (**100**) was of interest. Treatment of **66** with sulphuryl chloride in tetrachloromethane (Scheme VII) not only gave the required substitution product **100** but also a low yield of the subsequent addition product 2,3,3-trichloroflavanone (**101**).

Cytotoxicity and enzyme inhibition

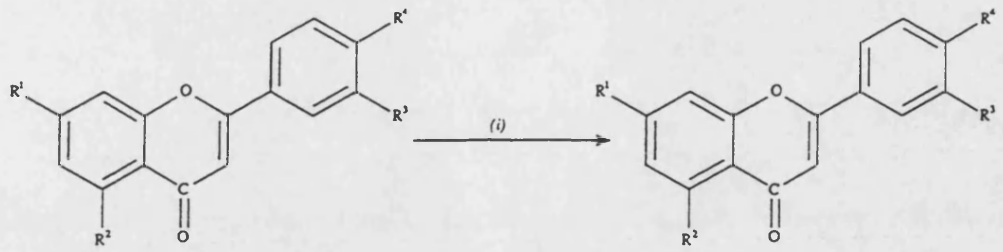
The ANN-1 cell line (Scher & Siegler, 1975) was used for an initial screen of inhibition of growth. Compounds were incubated for 3 days with the test flavones (50 μM). Those flavones which caused >50% inhibition of growth of ANN-1 cells at this concentration were tested for inhibition of growth of the parent cell line 3T3. This arbitrary initial test concentration was chosen to select those flavones showing potency in the same range as the tyrphostins (Gazit *et al.*, 1989). Compounds **77**, **78** and **89** were insufficiently soluble to be assayed at 50 μM and were shown to have no growth inhibitory effects at

Table III Yields and properties of flavones

Starting material	*Flavone*	*Method*	*Recrystallization solvent*	*Yield (%)*	*m.p. (°C)*	*Elemental analysis*			
						C (%)	*H (%)*	*N (%)*	
47	66	F	hexane	70	94[a]				
48[b]	67	F	hexane	72	106–107[c]				
49	68	F	EtOAc	73	115[d]				
50	69	F	hexane	64	161[e]				
51	70	F	EtOAc	60	132–133[f]				
52	71	F	EtOAc	72	242[g]				
53	72	F	$EtOCH_2CH_2OH$/ water	71	197[h]				
54	73	F	EtOAc	73	192–193[i]				
55	74	F	hexane	71	184.5–185.5[j]				
56	75	F	hexane	63	217	61.86	2.83		calc'd
						62.11	2.83		found
57	76	F	hexane	59	221[k]				
58	77	F	EtOAc	95	229	64.64	3.72	4.71	calc'd
						64.58	3.73	4.89	found
59	78	F	hexane	82	241	64.64	3.72	4.71	calc'd
						64.61	3.59	4.74	found
60	79	F	hexane	64	128[l]				
61	80	F	$CHCl_3$/ hexane	64	218	64.64	3.72	4.71	calc'd
						64.61	3.90	4.75	found
62	81	F	hexane	63	151[m]				
63	82	F	hexane	62	110[n]				
64	83	F	hexane	73	163	77.18	4.12		calc'd
						76.91	4.11		found
68	85	G	–	61	233–235[o]				
70	86	H	EtOAc	60	202[p]				
77	87	G	–	53	196	[q]			
78	88	G	–	48	217	[r]			

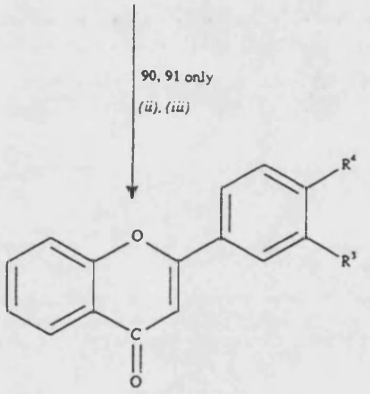
76	89	I	$EtOCH_2CH_2OH$/ water	62	219–220	65.8	4.5	9.0	calc'd
						65.7	4.4	8.6	found
71	90	J	EtOAc	56	224[s]				
72	91	J	EtOAc	62	237	75.93	4.67	5.90	calc'd
						75.65	4.62	5.95	found
76	92	J	EtOAc	62	231–232[t]				
77	93	J	EtOAc	47	181	71.89	4.90	5.24	calc'd
						71.91	5.04	5.53	found
78	94	J	EtOAc	75	189–190	71.89	4.90	5.24	calc'd
						71.61	5.02	5.20	found
89	95	K	–	85	oil	[u]			
90	96	L	MeOH	63	143	68.42	3.44	15.96	calc'd
						68.13	3.40	16.06	found
91	97	L	MeOH	76	105	68.42	3.44	15.96	calc'd
						68.14	3.35	16.25	found
84[v]	98	M	EtOH	76	237[w]				
66	99	N	EtOAc	86	165[x]				
66	100	O	–	83	123–124[y]				
66	101	O	–	1.5	102–104[z]	54.99	2.77		calc'd
						54.89	3.01		found

[a] lit. (Furniss *et al.*, 1981) m.p. 98°C
[b] Crude material prepared by Method D
[c] lit. (Chen *et al.*, 1962) m.p. 101–102°C
[d] lit. (Cavill *et al.*, 1954) m.p. 155°C
[e] lit. (Looker & Hanneman, 1962) m.p. 156–157°C
[f] (Looker & Hanneman, 1962) m.p. 130–131°C
[g] lit. (Doyle *et al.*, 1948) m.p. 244–246°C
[h] lit. (Reichel & Hempel, 1959) m.p. 201–202°C
[i] lit. (Baker *et al.*, 1952) m.p. 188–189°C
[j] lit. (Lin *et al.*, 1963) m.p. 178°C
[k] lit. (Jucker & Vogel, 1963) m.p. 227°C
[l] Compound reported by Lin *et al.* (1963) but no m.p. given
[m] lit. (Jain *et al.*, 1965) m.p. 153°C
[n] lit. (Ashihara *et al.*, 1977) m.p. 107–108°C
[o] lit. (Jain *et al.*, 1965) m.p. 243°C
[p] lit. (Looker & Hanneman, 1962) m.p. 209°C
[q] *m/z* 283.0485 (M^+) (100%) ($C_{15}H_9NO_5$ requires 283.1479)
[r] *m/z* 283.0490 (M^+) (100%) ($C_{15}H_9NO_5$ requires 283.1479)
[s] lit. (Gowan & Wheeler, 1950) m.p. 233°C
[t] lit. (Jucker & Vogel, 1963) m.p. 225°C
[u] *m/z* 280.1211 (M^+) ($C_{15}H_9NO_5$ requires 280.1211)
[v] Crude material prepared from 46 by Method D and Method F
[w] lit. (Simpson & Beton, 1954) m.p. 238–240°C
[x] lit. (Mann *et al.*, 1979) m.p. 169°C
[y] lit. (Mann *et al.*, 1979) m.p. 122–124°C
[z] lit. (Merchant & Rege, 1970) m.p. 114–115°C



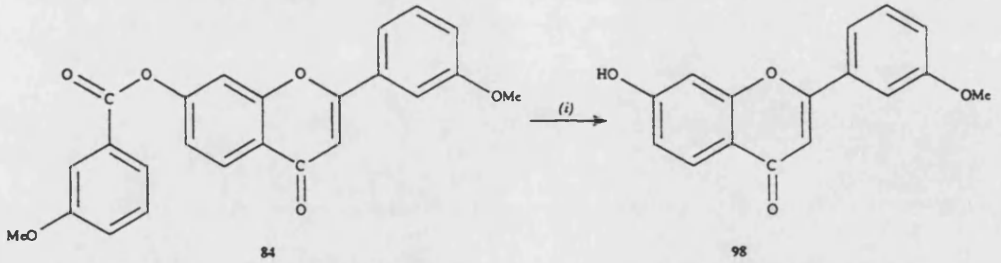
71: R^1, R^2, R^3 = H; R^4 = NO_2
72: R^1, R^2 = H; R^3 = NO_2; R^4 = H
76: R^1, R^2 = H; R^3 = NO_2; R^4 = Cl
77: R^1, R^2 = H; R^3 = NO_2; R^4 = OMe
78: R^1, R^2 = H; R^3 = OMe; R^4 = NO_2
89: R^1, R^2 = H; R^3 = NO_2; R^4 = NMe_2

90: R^1, R^2, R^3 = H; R^4 = NH_2
91: R^1, R^2 = H; R^3 = NH_2; R^4 = H
92: R^1, R^2 = H; R^3 = NH_2; R^4 = Cl
93: R^1, R^2 = H; R^3 = NH_2; R^4 = OMe
94: R^1, R^2 = H; R^3 = OMe; R^4 = NH_2
95: R^1, R^2 = H; R^3 = NH_2; R^4 = NMe_2



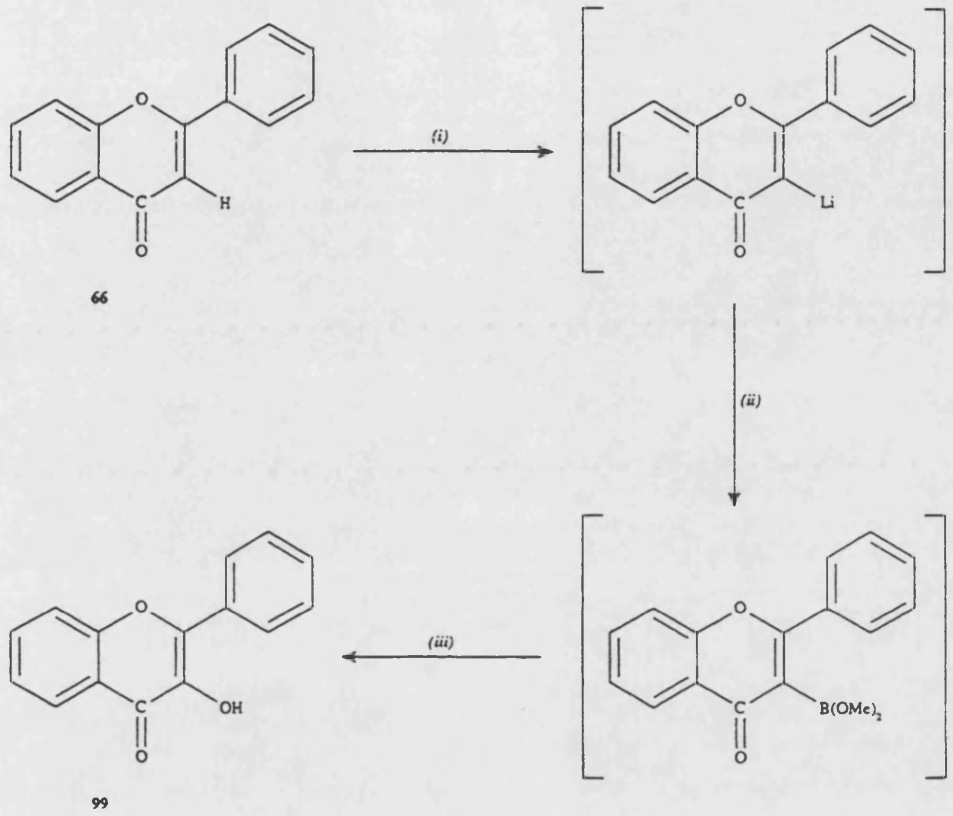
96: R^3 = H; R^4 = N_3
97: R^3 = N_3; R^4 = H

Scheme IV Reduction of nitroflavones 71, 72, 76–78 and 89 to give aminoflavones 90–95, and formation of azidoflavones 96 and 97. Reagents: (i) $SnCl_2$/HCl/EtOH; (ii) $NaNO_2$/HCl aq.; (iii) NaN_3



84 98

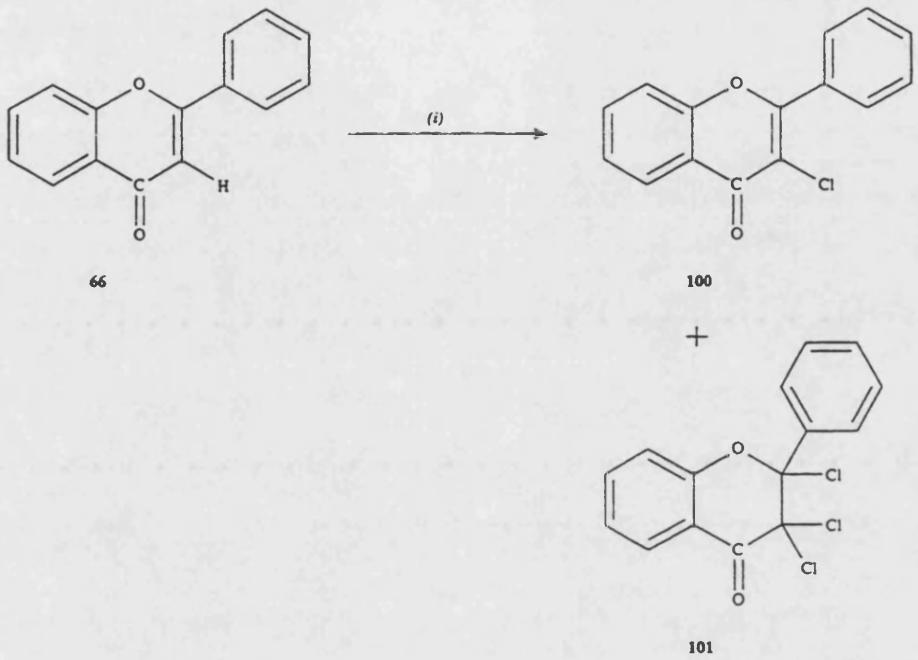
Scheme V Hydrolysis of ester 84 to give 7-hydroxy-3′-methoxyflavone (98). Reagent: (i) KOH aq



Scheme VI Synthesis of 3-hydroxyflavone (**99**). Reagents: (i) $LiNPr^{i}_{2}$/tetrahydrofuran; (ii) $(MeO)_3B$; (iii) H_2O_2/HOAc

7-methoxyflavone (**82**) were inhibitory to the Abelson kinase, both with kinetics which showed competition with ATP (data not shown). Interestingly, the antitumour agent flavone-8-acetic acid (**9**; Chart I) was a weak competitive inhibitor of this kinase. The other compounds tested, flavones **93** and **100**, showed no inhibition at concentrations <200 μM. The activity of the five inhibitors of the Abelson kinase was also measured against bovine brain cAMP-dependent protein kinase. No inhibition of the latter enzyme was observed at concentrations up to 200 μM; thus, inhibition of the tyrosine kinase is selective as defined by Graziani *et al.* (1983) in their studies of $pp60^{v\text{-}src}$. The abilities of the flavones to inhibit autophosphorylation were not studied.

Clearly, the inhibition of growth of Abelson-transformed ANN-1 cells appears not to result from an inhibition of the oncogene tyrosine kinase *abl*; for example, the most potent and selectively cytotoxic compound, **93**, was devoid of activity against the enzyme (Table VI). However, when the inhibitors of the Abelson kinase, together with compound **93**, were assayed against another protein tyrosine kinase—the EGF receptor-associated protein tyrosine kinase—**93** was found to inhibit its activity with a potency similar to that of quercetin (**7**) (Table VII). The existence of a relationship between inhibition of the EGF receptor protein tyrosine kinase and inhibition of growth of cells is



66 100 101

Scheme VII Chlorination of flavone (**66**). Reagent: (i) SO_2Cl_2/CCl_4

Table V Activities of flavones and analogous compounds as cytotoxic agents *in vitro*, comparing activity against 3T3 cells with activity against their Abelson-transformed counterparts, ANN-1 cells

Compound number[a]	*Compound name*[a]	*IC_{50} versus ANN-1 cells (range in μM)*	*IC_{50} versus 3T3 cells (range in μM)*
1	genistein	9–15	17–31
7	quercetin	22–43	29–50
66	flavone	100–140	22–35
67	3′-fluoroflavone	25–40	n.d.[b]
68	3′,4′-dimethoxyflavone	23–35	23–36
70	3′-methoxyflavone	18–30	n.d.[b]
75	3′,4′-dichloroflavone	5–20	58
81	5,7-dimethoxyflavone	22–30	22
82	7-methoxyflavone	45	n.d.[b]
85	3′,4′-dihydroxyflavone	8–16	19–26
86	3′-hydroxyflavone	14–35	21–37
90	4′-aminoflavone	10–20	18
91	3′-aminoflavone	24–32	20
92	3′-amino-4′-chloroflavone	8–27	12–25
93	3′-amino-4′-methoxyflavone	0.8–2.5	8
94	4′-amino-3′-methoxyflavone	20–35	38–54
99	3-hydroxyflavone	8–15	7–17

[a] Flavones **9**, **69**, **71–74**, **79**, **83**, **87**, **88**, **96**, **97** and **100** were not toxic to ANN-1 cells at 50 μM and/or were not soluble at this concentration

[b] n.d. = not determined

Table VI Activities of flavones against the tyrosine kinase activity of p*tabl*50; competition with ATP. Data are from experiments with purified protein

Compound number	*Compound name*	*Concentration of flavone (μM)*	K_m *(μM)*	K_i *(μM)*
7	quercetin	10	91	3.67
9	flavone-8-acetic acid	150	50	145
82	7-methoxyflavone	100	67	58
93	3'-amino-4'-methoxyflavone	500	–[a]	–[a]
99	3-hydroxyflavone	150	87	39
100	3-chloroflavone	360	–[a]	–[a]

[a] No activity observed at this concentration

Table VII Inhibition of the protein tyrosine kinase activity of EGF receptor derived from A431 cells by flavones

Compound number	*Compound name*	*Concentration of flavone (μM)*	*Percent inhibition*
7	quercetin	50	25 ± 16
9	flavone-8-acetic acid	150	−5 ± 9
93	3'-amino-4'-methoxyflavone	50	42 ± 11
99	3-hydroxyflavone	150	43 ± 11
100	3-chloroflavone	150	32 ± 9

being investigated. Cushman *et al.* (1991) reported that a series of 4'-aminoflavones inhibited the activity of the protein tyrosine kinase $p56^{lck}$ and it may be that our compounds inhibit growth via inhibition of some other protein tyrosine kinase. The findings from the above experiments suggest that a panel of tyrosine kinases might usefully be used to detect activity of candidate inhibitors, despite our use of the kinase responsible for the transformation of the primary screen, the ANN-1 cell line. It is clear that the assessment of the biological activity of tyrosine kinase inhibitors may be difficult. Faaland *et al.* (1991) showed that a tyrphostin could inhibit EGF-induced cytostasis in A431 cells, yet did this by a mechanism which appeared to be other than inhibition of the EGF-stimulated protein tyrosine kinase. We are pursuing the mechanism of action whereby the compounds which demonstrate antiproliferative effects might exert their activity.

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PUBLICATION 51

2-Nitroimidazole Dual-Function Bioreductive Drugs: Studies on the Effects of Regioisomerism and Side-Chain Structural Modifications on Differential Cytotoxicity and Radiosensitization by Aziridinyl and Oxiranyl Derivatives

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2-Nitroimidazole Dual-Function Bioreductive Drugs: Studies on the Effects of Regioisomerism and Side-Chain Structural Modifications on Differential Cytotoxicity and Radiosensitization by Aziridinyl and Oxiranyl Derivatives

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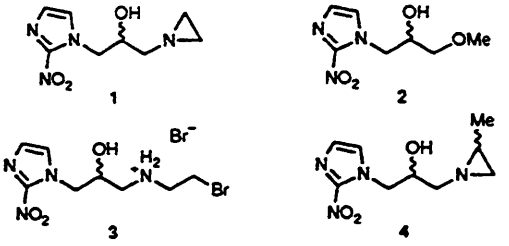
A series of 2-nitroimidazoles bearing side chains terminating in or containing aziridinyl and oxiranyl groups has been prepared, and the compounds were evaluated in vitro as hypoxia-selective bioreductively-activated cytotoxins and selected compounds tested for their radiosensitizing properties toward hypoxic mammalian cells. Compounds were either the regioisomers of analogues of the potent dual-functional 2-nitroimidiazole α-[(1-aziridinyl)-methyl]-2-nitro-1*H*-imidazole-1-ethanol (RSU-1069, 1) with additional methyl groups or related oxiranes of varying side-chain length and type. Oxiranyl derivatives showed little differential toxicity, and those tested were less effective as radiosensitizers, and although these properties were influenced by side-chain length, differences were not great. Aziridinyl compounds related to 1 but with increased side-chain lengths were unstable. Methylation of 1 in various regions had little effect on radiosensitization and no clear advantages over 1 as differential cytotoxic drugs. Progressive methylation at C-3 was found to increase toxicity but decrease hypoxia selectivity. Incorporation of a cyclohexane side chain in 1,2-*cis*-2,3-*trans*-3-aziridin-1-yl-2-hydroxy-1-(2-nitroimidazol-1-yl)cyclohexane (26) abolished hypoxia-selective toxicity and unexpectedly reduced radiosensitizing efficiency. Of the aziridines, 1-(2-nitro-1-imidazolyl)-2-methyl-3-(1-aziridinyl)-2-propanol (20) was comparable in efficacy to 1 as a bioreductively-activated cytotoxin with slightly lower aerobic toxicity; however, the prodrugs of 1 remain as preferred candidates for clinical evaluation.

Introduction

α-[(1-Aziridinyl)methyl]-2-nitro-1*H*-imidazole-1-ethanol (RSU-1069, 1) is the most effective of a large number of nitroheterocyclic compounds that have been synthesized and evaluated as radiosensitizers and bioreductively-activated cytotoxins.[1-3] Compounds of this type owe their selective cytotoxicity toward hypoxic cells within tumors to their bioreductive properties. Activation in vivo is by anaerobic enzymatic reduction to toxic metabolites.[4] The radiosensitizing activity of 2-nitroimidazoles is via fast free-radical mechanisms and is related to their one-electron reduction potentials.[5]

Compound (1) is an alkylating analogue of misonidazole (2)—a drug which failed clinically due to neurotoxicity.[6,7] Preliminary clinical investigation of 1 has, however, revealed gastrointestinal toxicity, which is severely dose-limiting.[8] A large number of other nitroheterocyclic moieties have been evaluated as carriers of aziridinyl and other alkylating functionalities; however, these have failed to produce comparable efficacy to the 2-nitroimidazoles as hypoxia-selective cytotoxins and any increase in radiosensitizing potency has generally been accompanied by higher toxicity.[9-12] Other approaches have been to reduce the observed toxicity of 1, and this has been achieved most effectively with the prodrug α-[[(2-bromoethyl)amino]-methyl]-2-nitro-1*H*-imidazole-1-ethanol (RB6145, 3), which is the current candidate for clinical evaluation.[13,14] With the exception of substitution of the aziridine ring, which results in lower toxicity, but also a reduction in selective bioreductive differential toxicity, there have been no reports of 2-nitroimidazoles closely related to 1 or its substituted analogues such as α-[(2-methylaziridinyl)-methyl]-2-nitro-1*H*-imidazole-1-ethanol (RSU-1131, 4).[2] We therefore present here the synthesis and evaluation of two classes of potentially alkylating 2-nitroimidazoles. (i) Aziridines: Regioisomers of 4 in which methyl substitution of the propyl side chain has been carried out are of interest to evaluate the steric influence of methyl substitution, which might be expected to influence reductase enzyme recognition and/or DNA binding. Such substituents may also be expected to influence the pK_a and consequent reactivity of the aziridine moiety. A close analogue of 1

Chart I



(1) Adams, G. E.; Ahmed, I.; Sheldon, P. W.; Stratford, I. J. Radiation sensitization and chemopotentiation: RSU-1069 a compound more efficient than misonidazole in vitro and in vivo. *Br. J. Cancer* 1984, *49*, 571–577.
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(6) Asquith, J. C.; Watts, M. E.; Patel, K.; Smithen, C. E.; Adams, G. E. Electron-affinic sensitization. V. Radiosensitization of hypoxic bacteria and mammalian cells in vitro by some nitroimidazoles and nitropyrazoles. *Radiat. Res.* 1974, *60*, 108–118.
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† University of Bath.

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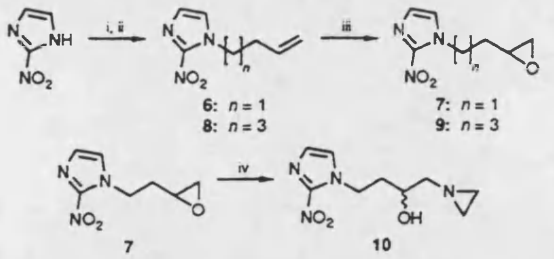
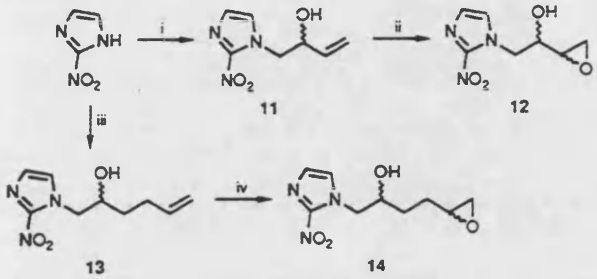
Scheme I[a]



[a] Reagents: (i) Bu^{t}OK/DMF, (ii) $Br(CH_2)_nCH_2CH{=}CH_2$, (iii) MCPBA/CH_2Cl_2, (iv) aziridine/EtOH.

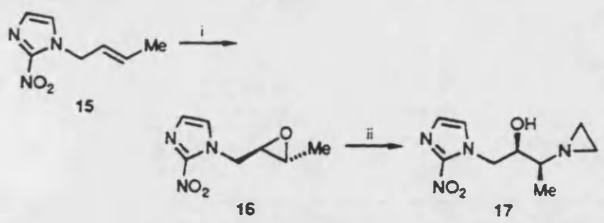
Scheme II[a]



[a] Reagents: (i) ethenyloxirane/DMF, (ii) MCPBA/CH_2Cl_2, (iii) 4-butenyloxirane, (iv) MCPBA/$CHCl_3$.

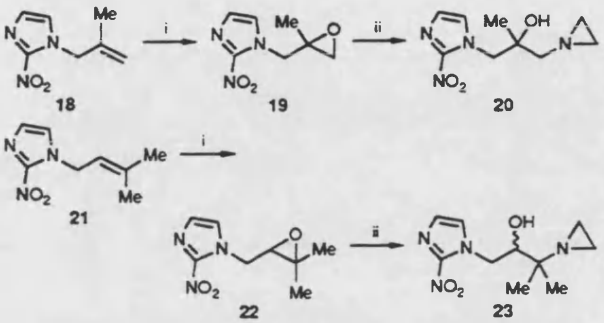
in which the 1-aziridinyl and 2-hydroxy groups are fixed in an antiperiplanar (trans-diaxial) conformation has also been synthesized by utilizing a cyclohexyl side chain in order to gain further information on structure–activity within this series, in particular steric and conformational effects. (ii) Oxiranes: One further way of moderating the toxicity of such alkylating 2-nitroimidazoles is to introduce

Scheme III[a]



[a] Relative stereochemistry only is shown. Reagents: (i) MCPBA/CH_2Cl_2, (ii) aziridine/EtOH/Et_3N.

Scheme IV[a]



[a] Reagents: (i) MCPBA/CH_2Cl_2, (ii) aziridine/EtOH/Et_3N.

a somewhat less reactive alkylating function. There have been no data published to date on differential toxicities of corresponding oxiranes although some radiosensitization data is available,[15] and we have therefore synthesized and evaluated a number of closely related and previously unreported oxiranyl analogues of varying side-chain lengths, and including the desoxy derivatives, which have been studied in view of recent reports that a hydroxyl group β to the imidazole is not required for activity in this class of bioreductive agent.[16,17]

Synthetic Chemistry

All compounds were synthesized from the common precursor 2-nitroimidazole (azomycin), which was available commercially in bulk quantity. This material was best utilized as its potassium salt in DMF, often in the presence of a crown ether. The alkenylnitroimidazoles **6** and **8** were thus synthesized from appropriate bromoalkenes in DMF with 18-crown-6 (Scheme I), whereas the 2-hydroxyalkenyl derivatives **11** and **13** were synthesized from 2-nitroimidazole and the appropriate epoxyalkene in refluxing DMF (Scheme II).

Reaction of oxirane **7** with refluxing aziridine in ethanol proceeded to give the chain-extended version of **1**, but this compound (**10**) was too unstable to isolate in a satisfactorily pure form for biological evaluation. Oxiranes **9**, **12**, and **14** gave only complex darkened mixtures under these

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(12) Naylor, M. A.; Stephens, M. A.; Stratford, I. J.; Keohane, A.; O'Neill, P.; Threadgill, M. D.; Webb, P.; Fielden, E. M.; Adams, G. E. Aziridinyl nitropyrroles and nitropyrazoles as hypoxia-selective cytotoxins and radiosensitizers. *Anti-Cancer Drug Des.* **1991**, *6*, 151–167.
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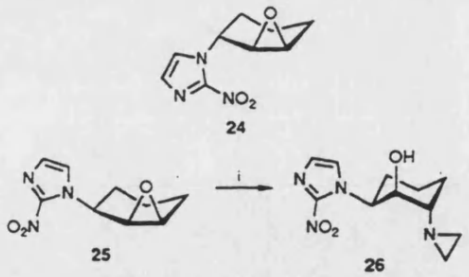
Scheme V[a]



[a] Reagents: (i) aziridine/EtOH/Et_3N.

and a variety of other conditions in the presence of aziridine.

Regioisomers of 4 and derivatives with further methyl substitutions in the hydroxypropyl side chain were synthesized via appropriately substituted oxiranes, which were themselves target compounds. Thus, as for the above compounds, appropriately substituted bromoalkenes were treated with potassium 2-nitroimidazole in DMF, and the resulting alkenes epoxidized with MCPBA to give the oxiranes **16**, **19**, and **22** (Schemes III and IV). Oxirane **16** was shown by NMR to be a mixture of isomers (approximately 10:1 trans:cis) corresponding to the isomeric content of the starting material. Oxiranyl synthons **16**, **19**, and **22** reacted cleanly with aziridine in refluxing ethanol containing 1% triethylamine to afford aziridines **17**, **20**, and **23**. Some selectivity was evident in the reaction of aziridine with the 2,3-disubstituted oxirane **16** in that **17** comprised an inseparable mixture of *erythro* and *threo* diastereoisomers in the ratio 4.5:1, as shown by ^1H NMR. Assignments and connectivity were established by a COSY experiment; the spectroscopic dispersion was sufficient at 250 MHz to permit measurement of most coupling constants for both stereoisomers. Karplus analysis of these data suggested that, in the predominant conformation for both *erythro*-**17** and *threo*-**17**, the aziridinyl group was gauche to the OH, in contrast to the conformation of compound **26** below.

The correct regioisomeric identity of compound **23** was established by the observation of a 6.2-Hz doublet for the OH proton in the 270-MHz NMR spectrum. This shows that the alcohol is secondary rather than tertiary and that the structure is as shown in Scheme IV rather than the regioisomer. Thus, it is demonstrated that the aziridine nucleophile has attacked the oxirane **22** at the more substituted carbon atom under basic conditions, in contrast to the regioselectivity usually observed for nucleophilic attack on substituted oxiranes. This regioselectivity may be the result of an electronic effect of the neighboring 2-nitroimidazole moiety, favoring ring opening at the distal epoxide carbon. The (2,3-epoxycyclohexyl)-2-nitroimidazoles **24** and **25** were synthesized as previously described.[18] The diastereoisomer **24** could be isolated in pure form for biological evaluation by virtue of its unreactivity toward nucleophilic attack by aziridine. Epoxide **25** reacts to give the corresponding aziridinyl derivative **26** (Scheme V).

Biological Evaluation in Vitro

Selective toxicity to hypoxic V79-379A cells was determined for all compounds using the MTT assay as has been described elsewhere.[12,19] These results are presented in Tables I and II, where C_{50}(air), the concentration required to kill 50% of the aerobic cells under the conditions of the assay, are divided by $C_{50}(N_2)$ values, concentrations required to kill hypoxic cells, to give differential toxicity ratios, which enable quantitative comparisons of bioreductive activities of drugs.

Table I. Biological Data for Oxiranyl 2-Nitroimidazoles

compd no.	$C_{1.6}$ (mM)[a]	C_{50}(air) (mM)[b]	$C_{50}(N_2)$ (mM)[c]	ratio[d]
5	0.25[e]	0.55	0.27	2.0
7	nd[f]	0.5	0.25	2.0
9	nd	1.2	0.7	1.7
12	0.2	1.2	0.36	3.3
14	0.4	0.6	0.4	1.5
16	nd	10.0	3.3	3.0
19	nd	0.2	0.2	1.0
24	nd	0.28	0.18	1.5

[a] Concentration required to give enhancement ratio = 1.6. [b] Concentration required to kill 50% of aerobic cells. [c] Concentration required to kill 50% of cells under N_2. [d] C_{50}(air)/$C_{50}(N_2)$. [e] Data taken from ref 15. [f] Not determined.

Table II. Biological Data for Aziridinyl 2-Nitroimidazoles

compd no.	$C_{1.6}$ (mM)[a]	C_{50}(air) (mM)[b]	$C_{50}(N_2)$ (mM)[c]	ratio[d]
1	0.1	0.3	0.015	20.0
4	0.08	0.79	0.065	12.2
17	0.12	0.2	0.01	20.0
20	nd[e]	0.55	0.02	27.5
23	0.07	0.1	0.008	12.5
26	0.45	0.35	0.35	1.0

[a] Concentration required to give enhancement ratio = 1.6. [b] Concentration required to kill 50% of aerobic cells. [c] Concentration required to kill 50% of cells under N_2. [d] C_{50}(air)/$C_{50}(N_2)$. [e] Not determined.

The oxiranyl derivatives **5**, **7**, **9**, **12**, **14**, **16**, **19**, and **24** showed only small additional toxicities as a result of bioreduction, and were indeed less effective than **2**. The high aerobic toxicity of oxiranes has also limited the bioreductive potential of other nitroheterocyclic compounds such as pyrroles and furans.[10,12] Those compounds that do not show high aerobic toxicity, such as **12** and **16**, are therefore likely to be limited in hypoxia selectivity by the reduced alkylating potential of the oxirane ring compared to the aziridine ring of their close analogues **1** and **17**, under similar conditions. Of the oxiranes most closely related to **1**, the four-carbon side chain of **12** resulted in greater differential cytotoxicity than a six-carbon side chain (**14**), although both differential values were low. A similar comparison can be made between compounds **7** and **9**. A side-chain length study of aziridinyl derivatives was not possible due to the instability of chain-extended versions of **1**, but these results indicate that elongation of the side chain does not improve the bioreductive potential. In contrast, studies on 2-nitroimidazoles with varying N-1 side-chain lengths terminating in morpholine, piperidine, and pyrrolidine showed that changes in sensitizing efficiency and, in some cases, cytotoxicity can occur.[20]

Aziridinylnitroimidazoles **17**, **20**, and **23** displayed differential cytotoxicities in the range 15–25; these figures are considered insignificantly different from values obtained

(18) Threadgill, M. D.; Webb, P. *syn*-Selectivity in the epoxidation of 1-(cyclohex-2-enyl)-2-nitroimidazole mediated by hydrogen bonding. *J. Chem. Soc., Chem. Commun.* **1991**, *4*, 269–271.

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for 1. Methyl substitution of the hydroxypropyl side chain therefore has little influence on bioreductive activity. Only 23 shows a more significant drop in activity, but more notable is the increase in aerobic toxicity in this example. Indeed the results for 1, 17, and 23 indicate progressive methylation at C-3 of the side chain increases toxicity in air and decreases hypoxia selectivity as a result. This could be due to progressive increases in the aziridine pK_a and hence increases in its reactivity to impractical levels. Consequently compound 23 has similar differential toxicity to the derivative containing a substituted aziridine (4), although this compound is known to contain a less potent alkylating group. All bioreductive activity was abolished when the side-chain stereochemistry was fixed in the trans-diaxial conformation of a cyclohexane substituent in compound 26. Aerobic toxicity was at a level attributable to the aziridine ring, with no additional toxicity under hypoxic conditions. Stereochemical and steric constraints on reductase enzyme recognition and binding are therefore in evidence and a cyclohexane skeleton will not be tolerated.

Aziridinyl-2-nitroimidazoles 1, 4, 17, 23, and 26, together with oxiranyl derivatives 12 and 14, were assayed for their ability to sensitize hypoxic V79 cells in vitro to γ-radiation, carried out as described previously.[12,13] The results are expressed in Tables I and II as $C_{1.6}$ values, the concentration of nitroimidazole required to give an enhancement ratio of 1.6.

The two oxiranyl derivatives evaluated were the compounds most closely related to 1 and containing a β-hydroxy substituent on the side chain. There was a 2-fold difference in these compounds sensitizing efficiencies, with 12, containing a 4-carbon side chain, being the more effective. This indicates that the chain length for maximum potency as a dual-function sensitizer may be quite small (four or five carbon atoms).

There was little effect on sensitizing efficiency within the series of aziridinyl derivatives upon methyl substitution of either the side chain or aziridine ring, although a similar small enhancement in sensitization was observed with compounds 4 and 23. Increases in aziridine pK_a as a result of these substitutions therefore may not be significant enough to improve tumor uptake, as has been observed with the more basic 2-nitroimidazoles including those bearing more substituted aziridines.[21] In contrast, the cyclohexyl derivative 26 was markedly less potent than its acyclic analogues. The contribution of the aziridine function toward radiosensitization has therefore been much reduced in 26 and its potency is even lower than that of non-alkylating nitroimidazoles such as 2. This is unexpected in view of the normal contribution the aziridine moiety exhibits toward aerobic toxicity in the bioreductive assay. The activity of 23 indicates that steric crowding at C-3 of the side chain is tolerated; hence the inactivity of 26 must be conformational in origin, not steric.

Conclusions

Although reduction of the alkylating potency of 1 by methyl substitution of the aziridine retains significant bioreductively-activated cytotoxicity, this is not the case for corresponding oxiranes. These derivatives do not have the alkylating potential required for bifunctional electrophilicity under hypoxic conditions and show little promise for further development.

Single methyl substitutions of the hydroxypropyl side chain of 1 has little effect on its activity, although aerobic toxicity was influenced by progressive C-3 methylations. No obvious advantages over 1 were apparent with these derivatives although 20 was slightly less toxic to aerobic cells and may be worthy of further study in vivo. Major structural modification of the side chain, for example with the cyclohexane ring of 26, abolishes activity, presumably by inhibiting bioreducibility. The prodrug 3 remains the candidate of this series of 2-nitroimidazoles for Phase I clinical evaluation.

Experimental Section

NMR spectra were obtained at 60 MHz with JEOL PMX60SI and at 270 MHz with JEOL GX270 spectrometers, using $SiMe_4$ as internal standard. Mass spectra were obtained with a VG 7070 spectrometer in either the electron impact (EI) mode at 70 eV or in the chemical ionizatioin mode (CI) with 2-methylpropane. Melting points are uncorrected. Elemental analyses were determined by Butterworth Laboratories Ltd., Teddington, Middlesex, U.K. Solutions in organic solvents were dried by treatment with Na_2SO_4 and filtration. Solvents were removed by evaporation under reduced pressure. Dichloromethane (CH_2Cl_2) was dried over calcium chloride and passed through neutral alumina prior to use. Dimethylformamide (DMF) and acetonitrile (CH_3CN) were anhydrous commercial grades. Silica gel radial chromatography was carried out on a Chromatotron (TC Research, Norwich, U.K.). 3-Chloroperbenzoic acid (MCPBA) either was the commercial grade (55%) or was purified by treatment with phosphate buffer (pH 7.0) and dried over P_2O_5 to obtain material of approximate 85% purity. 1-Potassio-2-nitroimidazole was obtained by treatment of azomycin with 1.1 equiv of potassium *tert*-butoxide (*t*-BuOK) in refluxing DMF, the mixture cooled, and the solid salt washed with CH_2Cl_2. Oxirane 5 was synthesized as described previously.[22] Compounds were racemic unless otherwise stated. Where elemental analyses and/or accurate mass spectra are not given due to compound form, instability, or lack of molecular ion, other mass spectral and NMR data were satisfactory and compounds were chromatographically homogeneous.

1-(3,4-Epoxybutyl)-2-nitroimidazole (7). 1-Potassio-2-nitroimidazole (3.03 g, 20 mmol) in dry CH_3CN (50 mL) was stirred with 4-bromo-1-butene (10 mL, 98.5 mmol) in the presence of 18-crown-6 (0.12 g, 0.45 mmol) for 16 days. The suspension was evaporated to dryness, redissolved in $CHCl_3$ (200 mL), and washed with H_2O (3 × 200 mL). The solution was then evaporated to dryness to afford 3.32 g (99%) of 6 as a pale yellow oil: NMR ($CDCl_3$) δ 2.55 (q, 2 H, J = 7.5 Hz, Im-CH_2CH_2CH=CH_2), 4.5 (t, 2 H, J = 7.5 Hz, Im-CH_2CH_2CH=CH_2), 5.3 (m, 3 H, 3 × olefinic-H), 7.1 (s, 1 H, Im-H), 7.25 (s, 1 H, Im-H). This material (3.32 g, 19.9 mmol) was dissolved in CH_2Cl_2 (100 mL) and MCPBA (55%, 7.3 g, 23.3 mol) added. The solution was heated under gentle reflux for 6 h, cooled, diluted with CH_2Cl_2 (100 mL), and washed with 10% Na_2SO_3(aq) (3 × 150 mL), H_2O (2 × 150 mL) and saturated $NaHCO_3$(aq) (2 × 150 mL). The solution was dried and evaporated, and the residue was purified on silica gel eluting with $CHCl_3$, to give 1.63 g (44.8%) of 1-(3,4-epoxybutyl)-2-nitroimidazole (7): mp 37–39 °C; NMR ($CDCl_3$) δ 2.0 (q, 2 H, J = 7.5 Hz, Im-CH_2CH_2), 2.4 (dd, 1 H, J = 3 and 4.5 Hz) and 2.9 (t, 1 H, J = 4.5 Hz) oxiranyl CH_2, 3.1 (m, 1 H, oxiranyl-H), 4.5 (t, 2 H, J = 7.5 Hz, Im-CH_2), 7.1 (s, 1 H, Im-H), 7.2 (s, 1 H, Im-H). Anal. ($C_7H_9N_3O_3$) C, H, N.

1-(5,6-Epoxyhexyl)-2-nitroimidazole (9). 1-Potassio-2-nitroimidazole (1.51 g, 10 mmol) in dry CH_3CN (50 mL) was stirred with 6-bromo-1-hexene (1.9 mL, 14 mmol) for 8 h in the presence of 18-crown-6 (0.1 g, 0.38 mmol). The solution was then heated under reflux for 12 h, cooled, and evaporated to dryness. The residue was redissolved in EtOAc (250 mL) and washed with H_2O (3 × 150 mL) and saturated NaCl(aq) (150 mL), dried, and evaporated to give 1.76 g (90%) of 1-(5-hexenyl)-2-nitroimidazole (8) as a yellow oil: NMR ($CDCl_3$) δ 1.8 (m, 6 H, Im-$CH_2(CH_3)_3$),

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4.5 (t, 2 H, J = 3.6 Hz, Im-CH_2), 5.25 (m, 3 H, CH=CH_2), 7.2 (br, 2 H, 2 × Im-H). This material (1.76 g, 9.0 mmol) was dissolved in CH_2Cl_2 (100 mL) and stirred with MCPBA (55%, 4.14 g, 13.2 mmol) at 25 °C for 6 h. A further 1.7 g (5.4 mmol) of MCPBA was then added and stirring continued for 16 h. The solution was diluted with CH_2Cl_2 (150 mL) and washed with 10% Na_2SO_3(aq) (3 × 150 mL), H_2O (2 × 150 mL), and saturated $NaHCO_3$(aq) (2 × 150 mL), dried, and evaporated. The residue was purified on silica, eluting with EtOAc, to afford 1.5 g (79%) of 9 as a pale yellow solid, recrystallized from aqueous ethanol: mp 29–31 °C; NMR ($CDCl_3$) δ 1.8 (m, 6 H, Im-$CH_2(CH_2)_3$), 2.5 (dd, 1 H, J = 3.6 and 4.8 Hz) and 2.9 (t, 1 H, J = 4.8 Hz), oxiranyl 6-CH_2, 3.1 (m, 1 H, oxiranyl 5-CH), 4.6 (t, 2 H, J = 7.5 Hz, Im-CH_2), 7.2 (s, 1 H, Im-H), 7.3 (s, 1 H, Im-H). Anal. ($C_9H_{13}N_3O_3$·0.25H_2O) C, H, N.

1-(2-Hydroxy-3,4-epoxybutyl)-2-nitroimidazole (12). 2-Nitroimidazole (3.0 g, 26.5 mmol) was dissolved in 10 mL of DMF and refluxed with 10 g (142 mmol) of butadiene monoxide and 0.2 g (1.4 mmol) of K_2CO_3. After 20 min the DMF was removed and the residue redissolved in H_2O (100 mL) and extracted with Et_2O (3 × 100 mL). The combined ether extracts were evaporated, and the residue was recrystallized from EtOAc/$CHCl_3$ to afford 2.5 g (52%) of 1-(2-hydroxy-3-butenyl)-2-nitroimidazole (11): mp 92–93 °C (lit.[23] mp 90–92 °C). This material (0.5 g, 2.7 mmol) was dissolved in 10 mL of dry CH_2Cl_2 together with 1.0 g (85%, 4.9 mmol) of MCPBA and the solution heated under reflux for 1.5 h. The solution was then cooled and evaporated, and the residue was purified on silica (eluting with EtOAc) to give 0.35 g (65%) of 12 as a pale yellow solid, recrystallized from EtOH: mp 131–132 °C (lit.[23] mp 134–136 °C); NMR ($(CH_3)_2SO$) δ 2.1 (t, 1 H, J = 2.4 Hz) and 2.7 (dd, 1 H, J = 2.4 and 4.8 Hz), oxiranyl 4-CH_2, 3.0 (m, 1 H, oxiranyl 3-CH), 3.9 (m, 2 H, C*H*OH), 4.8 (dd, 2 H, J = 3.6 and 7.5 Hz, Im-CH_2), 7.0 (s, 1 H, Im-H), 7.3 (s, 1 H, Im-H).

1-(2-Hydroxy-5,6-epoxyhexyl)-2-nitroimidazole (14). 2-Nitroimidazole (3.46 g, 30.6 mmol) and Na_2CO_3 (0.72 g, 6.8 mmol) were heated under reflux in DMF (35 mL) with 1,2-epoxy-5-hexene (3.75 mL, 33.2 mmol) for 15 min with stirring. The solution was then poured into EtOH (100 mL) and filtered, and the solid material was washed with EtOH (150 mL). The filtrate was evaporated to dryness and the residue redissolved in EtOAc (250 mL) and washed with H_2O (4 × 200 mL) and saturated NaCl(aq) (200 mL), dried, and evaporated. The residue was purified on silica, eluting with EtOAc, to afford 2.42 g (37.5%) of 1-(2-hydroxy-5-hexenyl)-3-nitroimidazole as a yellow solid (13): mp 81–83 °C; NMR ($CDCl_3$) δ 1.8 (dt, 2 H, J = 1.5 and 7.5 Hz, CH_2=$CHCH_2CH_2$), 2.4 (dt, 2 H, J = 2.4 and 7.5 Hz, CH_2=$CHCH_2CH_2$), 4.2 (br, 2 H, J = ca. 10 Hz, Im-CH_2), 4.35 (m, 2 H, C*H*OH), 5.7 (m, 3 H, CH_2=C*H*), 7.0 (s, 1 H, Im-H), 7.2 (s, 1 H, Im-H).

The above material (1.06 g, 5.3 mmol) was dissolved in $CHCl_3$ (100 mL) and 55% MCPBA (3.6 g, 11.5 mmol) added. The solution was stirred for 1 h at 25 °C and then heated under reflux for 1.5 h, cooled, and evaporated. The residue was purified on silica, eluting with EtOAc, to afford 0.83 g (73%) of 1-(2-hydroxy-5,6-epoxyhexyl)-2-nitroimidazole (14): mp 76–78 °C; NMR ($CDCl_3$) δ 1.8 (m, 4 H, CH_2CH_2-oxirane), 2.5 (dd, 1 H, J = 2.4 and 4.8 Hz) and 2.8 (t, 1 H, J = 4.8 Hz), oxirane 6-CH_2, 3.0 (m, 1 H, oxirane 5-CH), 4.35 (m, 4 H, Im-CH_2C*H*(O*H*)), 7.0 (s, 1 H, Im-H), 7.2 (s, 1 H, Im-H); CIMS, m/z = 228 (M + H).

***erythro*-1-(3-Aziridin-1-yl-2-hydroxybutyl)-2-nitroimidazole (17).** 1-Potassio-2-nitroimidazole (2.26 g, 15 mmol) and 1-bromo-2-butene (2 mL, 19.4 mmol) were refluxed in 20 mL of DMF for 5 min, cooled, poured into EtOH (100 mL), and filtered. The solid material was washed with hot EtOH (100 mL) and the filtrate evaporated, dried, and redissolved in CH_2Cl_2 (200 mL) and washed with H_2O (4 × 250 mL) and saturated NaCl(aq) (250 mL). The solution was dried and evaporated, and the residue was treated with activated charcoal (MeOH, reflux, 5 min), filtered, and evaporated to dryness to give 2.05 g (61.4%) of 1-but-2-enyl-2-nitroimidazole (15) as a yellow oil, which was used without further purification: NMR ($CDCl_3$) δ 1.8 (d, 3 H, J = 3.6 Hz, CH=CHC*H*$_3$), 5.1 (br d, 2 H, J = 4.8 Hz, Im-CH_2), 5.8 (m, 2 H, CH=CH), 7.15 (s, 1 H, Im-H), 7.2 (s, 1 H, Im-H).

The alkene 15 (0.84 g, 5.0 mmol) was dissolved in CH_2Cl_2 (50 mL) and MCPBA (55%, 1.7 g, 5.5 mmol) added. The solution was heated under reflux for 6 h, cooled, and diluted with CH_2Cl_2 (100 mL). The organic material was washed with 10% Na_2SO_3(aq) (2 × 100 mL), saturated $NaHCO_3$(aq) (2 × 150 mL), H_2O (2 × 150 mL), and saturated NaCl(aq) (200 mL), dried, and evaporated. The residue was purified by silica gel radial chromatography, eluting with CH_2Cl_2, to give 0.47 g (51.1%) of 1-(2,3-epoxybutyl)-2-nitroimidazole (16) as a pale yellow gum: NMR ($CDCl_3$) δ 1.3 (d, 3 H, J = 4.8 Hz, oxirane 3-CH_3), 2.8 (dd, 1 H, J = 2.4 and 4.8 Hz, oxirane 3-CH), 3.1 (m, 1 H, oxirane, 2-CH), 4.2 (dd, 1 H, J = 7.5 and 14.4 Hz), and 4.9 (dd, 1 H, J = 3.6 and 14.4 Hz), Im-CH_2, 7.1 (s, 1 H, Im-H), 7.15 (s, 1 H, Im-H); EIMS 183.0635 (calcd for $C_7H_9N_3O_3$ 183.0644).

The oxiranyl synthon 16 (0.46 g, 2.5 mmol) was dissolved in EtOH/1% Et_3N (20 mL) and heated under reflux with 1*H*-aziridine (CAUTION!) (2.0 g, 46.5 mmol) for 8 h. The solution was evaporated and the residue purified on silica (eluting with $CHCl_3$/MeOH/Et_3N, 98:1:1) to give, after recrystallization from EtOH (1% Et_3N), 0.46 g (81%) of 17 as an off-white solid: mp 113–114 °C; NMR ($CDCl_3$/$(CD_3)SO$) δ 1.3 (s, 3 H, CH(C*H*$_3$)), 1.35 (dd, 2 H, J = 2 and 4 Hz, aziridine-CH_2), 1.8 (dd, 2 H, J = 2 and 4 Hz, aziridine-CH_2), 2.6 (m, 1 H, C*H*(CH_3)), 4.0 (m, 1 H, C*H*-(OH)), 4.4 (dd, 1 H, J = 9.6 and 19.2 Hz) and 4.8 (dd, 1 H, J = 2.4 and 12 Hz), Im-CH_2, 7.1 (s, 1 H, Im-H), 7.3 (s, 1 H, Im-H). *erythro*-17: NMR ($CDCl_3$, 250 MHz) δ 1.17 (d, 3 H, J = 7 Hz, CH_3), 1.31 (ca. q, 1 H, J = ca. 4 Hz) and 1.37 (ca, q, 1 H, J = ca. 4 Hz), 1.67 (ca. q, 1 H, J = ca. 4 Hz) and 1.69 (ca, q, 1 H, J = ca. 4 Hz), aziridine-H_4, 1.54 (dq, 1 H, J = 3 and 7 Hz, C*H*CH_3), 3.7 (br 1 H, OH), 4.03 (dd, 1 H, J = 3 and 10 Hz, CHOH), 4.08 (dd, 1 H, J = 10 and 13 Hz) and 4.72 (d, 1 H, J = 13 Hz), imidazole-CH_2, 7.14 (s, 1 H, Im-4-H), 7.29 (s, 1 H, Im-5-H). *threo*-17: NMR δ 1.22 (d, 3 H, J = 7 Hz, CH_3), 1.37 (m, 1 H) and 1.40 (m, 1 H) and 1.90 (ca. q, 1 H, J = ca. 4 Hz) and 1.92 (ca. q, 1 H, J = ca. 4 Hz), aziridine-H_4, 1.66 (dq, 1 H, J = 6 and 7 Hz, C*H*CH_3), 3.6 (br, OH), 3.91 (ddd, 1 H, J = 2.5 and 6 and 10 Hz, C*H*OH), 4.26 (dd, 1 H, J = 10 and 14 Hz) and 4.87 (dd, 1 H, J = 2.5 and 14 Hz), imidazole-CH_2, 7.13 (br s, 1 H, Im-4-H), 7.27 (br s, 1 H, Im-5-H); CIMS m/z = 227 (M + H), 197, 180. Anal. ($C_9H_{14}N_4O_3$·0.25H_2O) C, H, N.

1-(2-Nitro-1-imidazolyl)-2-methyl-3-(1-aziridinyl)-2-propanol (20). To 1-potassio-2-nitroimidazole (5.72 g, 37.9 mmol) in 50 mL of DMF was added sodium iodide (0.1 g, 0.67 mmol) followed by β-methallyl chloride (10 mL, 101 mol) and the solution heated under reflux for 0.25 h. The solution was then cooled, poured into EtOH (100 mL), and filtered, and the solid material was washed with warm EtOH (150 mL). The combined filtrates were evaporated, and the residue was redissolved in $CHCl_3$ (250 mL), washed with H_2O (4 × 200 mL) and saturated NaCl(aq) (200 mL), dried, and evaporated to afford 6.95 g (82.2%) of 1-(2-methylprop-2-enyl)-2-nitroimidazole (18) as a pale yellow oil, which was used without further purification: NMR ($CDCl_3$) δ 1.8 (s, 3 H, $CH_2C(CH_3)$), 4.6 (bs, 1 H) and 5.0 (bs, 1 H), $C(CH_3)$=CH_2), 5.0 (s, 2 H, Im-CH_2), 7.2 (s, 2 H, 2 × Im-H). This material (3.42 g, 20.5 mmol) was dissolved in CH_2Cl_2 (100 mL) and heated under reflux with MCPBA (55%, 12.14 g, 38.8 mmol) for 3 h. The cooled solution was diluted with $CHCl_3$ (150 mL) and washed with 10% Na_2SO_3(aq) (3 × 200 mL), saturated $NaHCO_3$(aq) (2 × 200 mL), H_2O (2 × 200 mL), and saturated NaCl(aq) (200 mL). The solution was dried and evaporated and the residue purified on silica, eluting with EtOAc, to give 1.89 g (50.4%) of 1-(2-methyl-2,3-epoxypropyl)-2-nitroimidazole (19) as a yellow solid: mp 70–71 °C; NMR ($CDCl_3$) δ 1.4 (s, 3 H, oxirane 2-CH_3), 2.7 (dd, 2 H, J = 3.6 and 14.5 Hz, oxiranyl 3-CH_2), 4.7 (dd, J = 14.5 and 33 Hz, Im-CH_2), 7.2 (s, 1 H, Im-H), 7.25 (s, 1 H, Im-H). Anal. ($C_7H_9N_3O_3$) C, H, N. The oxirane 19 (1.26 g, 6.9 mmol) was heated under reflux in EtOH (1% Et_3N, 30 mL) with 1*H*-aziridine (2.0 g, 46.5 mmol) for 0.75 h. The solvent was then evaporated and the residue recrystallized from EtOH (1% Et_3N) to afford 0.93 g (59.8%) of 20 as a pale yellow solid: mp 108–110 °C; NMR ($CDCl_3$/$(CD)_3SO$) δ 1.2 (s, 3 H, C(OH)C*H*$_3$), 1.25 (dd, 2 H, J = 2 and 4 Hz, aziridine CH_2), 1.8 (dd, 2 H, J = 2 and 4 Hz, aziridine CH_2), 2.3 (dd, 2 H, J = 12 and 22 Hz, CH_2-aziridine), 4.7 (s, 2

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H, Im-CH_2), 7.05 (s, 1 H, Im-H), 7.3 (s, 1 H, Im-H). Anal. ($C_9H_{14}N_4O_3$) C, H, N.

1-(2-Nitro-1-imidazolyl)-3,3-dimethyl-3-(1-aziridinyl)-2-propanol (23). 1-Potassio-2-nitroimidazole (3.05 g, 20.2 mmol) in dry CH_3CN (50 mL) was stirred with 4-bromo-2-methylbut-2-ene (4.75 g, 31.8 mmol) and 18-crown-6 (0.26 g, 0.98 mmol) for 24 h. The suspension was evaporated and redissolved in $CHCl_3$ (200 mL) and washed with H_2O (4 × 75 mL) and saturated NaCl (75 mL) and then dried and evaporated to dryness. The residue was purified on silica gel eluting with $CHCl_3$ to afford 0.8 g (22%) of 1-(3-methylbutenyl)-2-nitroimidazole (21) as a pale yellow oil: NMR ($CDCl_3$) δ 1.8 (bs, 6 H, $CH{=}C(CH_3)_2$), 5.1 (bd, 2 H, Im-CH_2), 5.4 (m, 1 H, $CH{=}C(CH_3)_2$), 7.1 (s, 1 H, Im-H), 7.15 (s, 1 H, Im-H).

The above alkene (2.7 g, 14.9 mmol) was dissolved in CH_2Cl_2 (100 mL) and heated under reflux with MCPBA (6.29 g, 20.1 mmol) for 1 h, cooled, diluted with CH_2Cl_2 (100 mL), and washed with 10% Na_2SO_3(aq) (3 × 150 mL), H_2O (2 × 150 mL), and saturated $NaHCO_3$(aq) (2 × 150 mL). The solution was dried (Na_2SO_4) and evaporated, and the residue was purified on silica, eluting with EtOAc, to give 1.8 g (81.7%) of 1-(3,3-dimethyl-2,3-epoxybutyl)-2-nitroimidazole (22) as a pale yellow oil: NMR ($CDCl_3$) δ 1.4 (s, 3 H, oxirane 3-CH_3), 1.45 (s, 3 H, oxirane 3-CH_3), 3.2 (dd, 1 H, J = 3.6 and 8.4 Hz, oxirane 2-CH), 4.3 (dd, 1 H, J = 8.4 and 13.5 Hz), and 5.1 (dd, 1 H, J = 3.6 and 14.4 Hz), Im-CH_2, 7.15 (s, 1 H, Im-H), 7.3 (s, 1 H, Im-H).

The oxiranyl synthon 22 (1.09 g, 5.5 mmol) was dissolved in EtOH (1% Et_3N) (30 mL) and heated under reflux with 1*H*-aziridine (2.0 g, 46.5 mmol) for 10 h. Further aziridine (5 mL, 116 mmol) was then added, and heating was continued for another 4 h. The solution was then evaporated and the residue purified on silica (eluting with $CHCl_3$/MeOH/Et_3N (90:9:1)) to afford, after recrystallization from EtOH (1% Et_3N), 0.32 g (32.8%) of 23 as a pale yellow solid: mp 127–128 °C; NMR ($(CD_3)_2SO$) δ 0.78 (s, 3 H, $C(CH_3)$), 0.83 (s, 3 H, $C(CH_3)$), 1.4 (m, 4 H, 2 × aziridine CH_2), 4.25 (dd, 1 H, J = 10.7 and 13.5 Hz) and 4.92 (dd, 1 H, J = 1.5 and 13.5 Hz), Im-CH_2, 5.07 (d, 1 H, J = 6.2 Hz, OH), 7.14 (s, 1 H, Im-H), 7.59 (s, 1 H, Im-H); NMR ($CDCl_3$) δ 0.92 (s, 3 H, $C(CH_3)$), 0.98 (s, 3 H, $C(CH_3)$), 1.5 (m, 2 H) and 1.53 (m, 1 H) and 1.61 (m, 1 H), 2 × aziridine CH_2, 3.66 (dd, 1 H, J = 1.7 and 10.1 Hz, $CHOH$), 4.10 (dd, 1 H, J = 10.2 and 13.5 Hz) and 4.9 (dd, 1 H, J = 1.7 and 13.5 Hz), Im-CH_2, 4.2 (br, 1 H, OH), 7.08 (d, 1 H, J = 1.1 Hz, Im-H), 7.27 (d, 1 H, J = 1.1 Hz, Im-H). Anal. ($C_{10}H_{16}N_4O_3$) C, H, N.

1,2-*cis*-2,3-*trans*-3-Aziridin-1-yl-2-hydroxy-1-(2-nitroimidazol-1-yl)cyclohexane (26). 1-Cyclohex-2-enyl-2-nitroimidazole was prepared and epoxidized with MCPBA as described previously.[18] The anti-isomer 24 was isolated as unreacted starting material when a mixture of isomers was used for the following reaction. The syn-isomer 25 reacts with aziridine, but could be obtained in pure form when the above epoxidation was carried out with dried peroxy acid as described previously.[18]

The oxiranyl synthon 25 (0.5 g, 2.4 mmol) was refluxed in 40 mL of EtOH (1% Et_3N) with 1*H*-aziridine (2.0 g, 46.5 mmol) for 1.5 h. The solution was evaporated to dryness and the residue purified on silica, eluting with $CHCl_3$/MeOH/Et_3N (90:9:1), to give 0.15 g (26%) of 26 as a pale yellow solid, recrystallized from acetone: mp 204–206 °C; NMR ($(CD_3)_2SO$) δ 1.12 (dd, 1 H, J = 6.0 and 3.3 Hz, aziridine-H syn to N–cycohexane bond), 1.16 (dd, 1 H, J = 6.0 and 3.4 Hz, aziridine-H syn to *N*–cyclohexane bond), 1.45–1.90 (m, 7 H, 2 × aziridine-H anti to *N*–cyclohexane bond + 4ax,4eq,5ax,5eq,6eq-H), 2.17 (dq, 1 H, J = 11.4 and 3.4 Hz, 6ax-H), 3.34 (m, 1 H, 3-H), 3.91 (m, 1 H, 2-H), 5.11 (d, 1 H, J = 5.6 Hz, OH), 5.49 (d ca. t, 1 H, J = 11.9 and 3 Hz, 1-H), 7.15 (d, 1 H, J = 0.7 Hz, Im-4H), 7.67 (d, 1 H, J = 0.7 Hz, Im-5H); EIMS 252.1222 (calcd for $C_{11}H_{15}N_4O_3$ 252.1240).

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Analysis of Cocaine, Benzoylecgonine, Ecgonine Methyl Ester, Ethylcocaine and Norcocaine in Human Urine using HPLC with Post-Column Ion-Pair Extraction and Fluorescence Detection

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Analysis of cocaine, benzoylecgonine, ecgonine methyl ester, ethylcocaine and norcocaine in human urine using HPLC with post-column ion-pair extraction and fluorescence detection*

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Abstract: The measurement of cocaine and its major metabolites has been achieved by an HPLC method that compensates for their different solubilities and detection properties. Although ecgonine methyl ester is a major metabolite it is generally not measured by HPLC because it is poorly detectable by UV, and its water solubility makes recovery from urine difficult. Using modified solid-phase extraction procedures recoveries of 85% for ecgonine methyl ester, 97% for cocaine, 106% for benzoylecgonine and 80% for ethylcocaine have been obtained from urine. Increased chromatographic retention and detection sensitivity has been obtained by formation of the *t*-butyldimethylsilyl derivative of ecgonine methyl ester which was found to be stable in the HPLC mobile phase for at least 1 week. Alkylation of norcocaine and benzoylecgonine has improved their detection sensitivity and also chromatographic resolution. All calibrations were linear over the range 200–1000 ng ml^{-1} in urine with correlation coefficients >0.99.

Keywords: *Cocaine; benzoylecgonine; ecgonine methyl ester; ethylcocaine; post-column high-performance liquid chromatography; silylation; alkylation.*

Introduction

The development of improved analytical procedures for drugs of abuse continues to be an active area of research because of the increasing availability of drugs world-wide. In this laboratory, sensitive and selective methods have been developed using pre-column derivatization [1], detection at 205 nm [2] and post-column ion-pair extraction [3] for drugs of abuse. Cocaine is widely abused for its CNS stimulant properties. In humans cocaine is metabolized and excreted in the urine mainly as benzoylecgonine (BE) and ecgonine methyl ester (EME). Typical values for cocaine excretion are BE 46%; EME 41%; cocaine 3% and the other metabolites about 10% [4]. In cases where cocaine is concurrently abused with ethanol an additional metabolite, ethylcocaine is produced [5]. Metabolism of cocaine by liver esterases forms EME and the resulting loss of the benzoyl group means that the metabolite has lost its important chromophore, has become more water soluble and is difficult to recover from urine using procedures suitable for cocaine or benzoylecgonine.

Numerous HPLC methods have been described for the measurement of cocaine and its metabolites [6, 7] but usually EME is not included, even in recent methods [8]. However, as a major metabolite of cocaine [9] EME is important in both drug metabolism studies and in forensic investigations since, with BE, it is an indication of when the cocaine was ingested. Examples of methods that measure EME include that by Miller and DeVane [10] who used an RP-HPLC procedure to measure C and BE by UV detection at 230 nm and also C and EME by electrochemical detection, with the two detectors arranged in series. A mobile phase of pH 8.8 was required to detect EME so that a polymeric column was required. Ethylcocaine was selected as internal standard, and the analytes measured in sheep plasma in the calibration range of 100–2400 ng ml^{-1}. GC with a nitrogen phosphorous detector has been used [11] to measure C, BE and EME in urine following SPE extraction and derivatization of BE and EME. Detection limits were 100 ng ml^{-1} for C and BE and 250 ng ml^{-1} for EME. Unfortunately, BE was derivatized to its ethyl ester, ethylcocaine and the percentage re-

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covery of EME was only 41%. GC–MS has been described [12] to measure C, BE, EME and EC in hair collected from cocaine users, and the presence of ethylcocaine found to be a particularly useful indication of cocaine use, since cocaine could conceivably contaminate the hair externally. In this work an HPLC method is reported for the simultaneous measurement of cocaine, benzoylecgonine, ecgonine methyl ester and ethylcocaine from urine by suitable modification of solid-phase extraction procedures, derivatizations to improve chromatography and extractions, followed by post-column ion-pair formation with fluorescence detection.

Experimental

Chemicals

Cocaine HCl was obtained from the dispensary of the School of Pharmacy and Pharmacology, University of Bath. Benzoylecgonine was synthesized following the procedure of Lampert and Stewart [6]. Ecgonine methyl ester was prepared from ecgonine following the procedure of Findlay [13]. Ecgonine was prepared by acid hydrolysis of cocaine. Ethylcocaine was prepared from ecgonine by treating it with iodoethane in the presence of potassium carbonate in acetone. Norcocaine was prepared following the procedure of Borne [14]. The structures of all the metabolites was confirmed by IR and ^{1}H NMR. The purity of the samples was confirmed by HPLC and for EME by GC. No extraneous peaks were observed.

Acetonitrile, methanol, ammonia, dichloroethane, dichloromethane, 9,10 dimethoxy anthracene sulphonate sodium salt (DAS) were HPLC grade and were obtained from Fisons (Loughborough, UK). Buffer components (analytical grade) were also obtained from Fisons. Iodoethane, iodobutane and iodohexane were synthesis grade (99% purity) and obtained from Aldrich Chemical Company (Gillingham, Dorset, UK). MTBSTFA (*N*-methyl-*N*{*tert*-butyldimethylsilyl}trifluoroacetamide) was obtained from Pierce Warriner Ltd (Cheshire, UK). Urine was Lyphocheck 'Screen Control Negative' from Bio-Rad Labs (Hemel Hempstead, UK). Water was distilled from an all glass still. Solid-phase extraction was carried out using a Vac Elut SP24 unit with Bond-Elut 'Certify' (300 mg) cartridges from Jones Chromatography (Hengoed, UK).

Instrumentation

Chromatography was performed using an SSI (State College, PA, USA) HPLC system consisting of a gradient system Model 402 with a microbore head that was modified to carry out gradients at low flow rates. Commercial low pressure gradient systems are designed to carry out gradient elution at flow rates of 1.0 ml min^{-1} or more with 4.6 mm i.d. columns. The volume of the mobile phase in the system, i.e. the gradient controller, the pump and the associated tubing before the column is about 5 ml and causes a delay in the time before the gradient reaches the column of about 5 min. If the same system is used at flow rates of 0.2–0.4 ml min^{-1}, as required for columns of 2.1 mm i.d., the delay time becomes 25–12.5 min. This delay also causes problems with the gradient pattern and so modifications are required to minimize this effect. This was achieved by using short 0.8 mm i.d. tubing to connect the pump to the gradient controller instead of the usual 2.0 mm i.d. tubing and using short narrow bore tubing (0.2 mm i.d.) between the column and the purge valve of the pump. These modifications reduced the delay time of 12.5 min to 4.0 min at 0.4 ml min^{-1}.

A Rheodyne 7125 model with a 50 μl loop in a model 505 oven and maintained at 40°C was connected to a 250 × 2.1 mm i.d. Supelco pKb 100 column, 5 μm particle size (Supelco, Bellefonte, PA, USA). The column was attached to the post-column ion-pair extraction detector as shown in Fig. 1, consisting of two model 350 pumps with either a Guardian pulse dampener or Model LP Lo-Pulse dampener (SSI). Additional pulse dampening was provided with 100 × 4.6 mm i.d. columns containing 10 μm Partisil silica (aqueous phase) and 10 μm CPS-Hypersil (organic phase). The addition of the aqueous reagent to the HPLC eluent and the segmentation of aqueous phase with organic phase was achieved using Tee-connectors, 1/16 × 0.015 inch (SSI). An Uptight precolumn, 20 × 2 mm i.d. (Anachem) packed with 75 μm glass Ballotini beads was used as mixing column. The extraction coil was 1.5 m × 0.8 mm i.d. s/s tubing. The phase separator, which has been described elsewhere [3] was connected to a Perkin–Elmer Model 204S Spectrofluorometer fitted with a 100 μl flow cell (Perkin–Elmer–Hitachi) and connected to a Servoger 120 chart recorder.

The mobile phase used an acetonitrile step

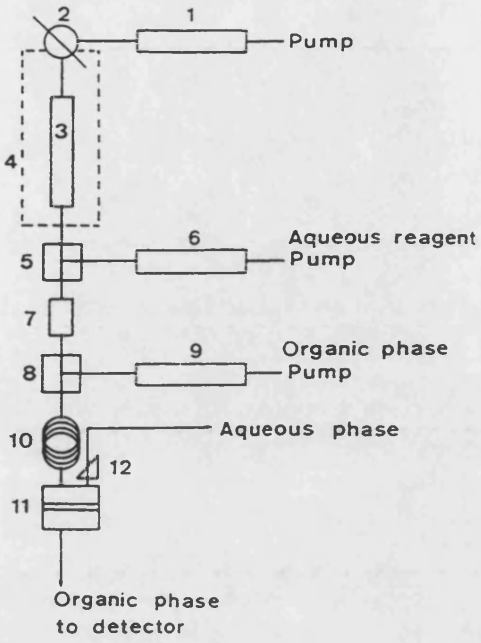

Figure 1
Schematic diagram of HPLC post-column system. 1,6 and 9 = in-line columns; 2 = injection valve; 3 = analytical column; 4 = oven; 5 and 8 = Tee connectors; 7 = mixing column; 10 = extraction coil; 11 = phase separator; and 12 = microneedle valves.

gradient from 11 to 30% v/v acetonitrile in 0.05 M phosphate buffer (pH 4.0) at 0.4 ml min^{-1} at 7.0 min after injection, and returned to 11% at 25 min. 9,10-dimethoxyanthracene sulphonate sodium salt (DAS, 34 mg l^{-1}) was added to the eluent flow post-column at a flow rate of 0.5 ml min^{-1}. Dichloroethane was used to segment and extract the drug–DAS ion-pairs at a flow rate of 0.6 ml min^{-1}. Fluorescence detection was achieved using an excitation wavelength of 383 nm and emission wavelength of 465 nm.

Extraction procedure

An appropriate amount of an acetonitrile stock solution of the various standards was pipetted into glass tubes, evaporated to dryness using nitrogen and 1.0 ml of Bio-Rad urine, prepared according to the manufacturer's instruction, was added to the dried residue. The sample was vortexed for 1 min and 4.0 ml of phosphate buffer (pH 6.0) added.

Bond-Elut 'Certify' LRC columns were inserted into a SP24 vacuum manifold and conditioned with 6.0 ml methanol and 6.0 ml phosphate buffer (pH 6.0). The columns were prevented from drying out at this stage by turning the vacuum off. The samples prepared above were poured into the prepared cartridges and gently pulled through. After this the cartridges were washed with 6.0 ml of water and air-dried for 5 min at 15 mm Hg. They were then washed with 9.0 ml of 0.1 N HCl followed by 7 ml methanol and 3 ml acetonitrile. Elution of the samples was performed with 6.0 ml of 1.5% NH_4OH in dichloromethane–isopropanol (80:20%, v/v). The samples were transferred to Reacti-vials (Pierce, Warrington, Cheshire, UK) and evaporated to dryness using a gentle stream of N_2 and derivatized.

Derivatization procedures

(a) The alkylation procedure was a modification of that described by Ortuno *et al.* [11] for the derivatization of benzoylecgonine and norcocaine. To the dried residue of standards, 180 μl of acetonitrile, 30 μl of iodobutane, 10.0 mg of K_2CO_3 and 20 μl of 0.20 mg ml^{-1} 18 Crown 6 ether in acetonitrile was added. The Reacti-vial was closed, vortexed for 30 s, and then heated at 85°C for 1 h. After the samples were allowed to cool they were evaporated to dryness under N_2.

(b) Silylation of EME. Acetonitrile (100 μl) and MTBSTFA (50 μl) were added to the dried residue from (a) and the vials heated at 60°C for 30 min. The samples were then allowed to cool and evaporated to dryness under a gentle stream of N_2. To the dried residue, internal standard (50 μl), mobile phase (350 μl) and 8.5% phosphoric acid (100 μl) were added and vortexed for 30 s. The sample was injected into the HPLC system. Peak height ratios were measured and plotted against concentration of standards. The recoveries of samples from urine were calculated by comparison with non-extracted standards.

Results and Discussion

Initial attempts with Bond-Elut 'Certify' cartridges (130 mg) following the manufacturer's instructions showed that recovery of EME from spiked urine was around 40%. Subsequent examination of the process showed that EME was being lost during the first washing procedure. To improve recoveries of EME the capacity of the cartridge was increased to 300 mg, and this raised recoveries to

70–80%. However this cartridge size also retained additional endogenous components from the urine. To clean the urine extract more thoroughly the volume of wash solvents and eluting solvents was also increased. Initially as recommended by the manufacturer methanol alone was used as the wash solvent but this lowered the recoveries of EME and ethylcocaine. Replacing methanol with acetonitrile gave a less clean blank chromatogram. A combination of 7.0 ml of methanol and 3.0 ml of acetonitrile was found to give the best compromise in terms of recoveries and clean blanks.

An additional complication was that some extra peaks were found to originate from the cartridge material itself and repeated washing of the cartridges with methanol or eluting solvents failed to remove them. The peaks were found to be proportional to the percentage of ammonia in the eluting solvent, as shown in Fig. 2, and is probably caused by ammonia attacking the silica-based packing material. Triethylamine had the same effect as ammonia. Decreasing the percentage of ammonia in the eluting solvent also lowered the recoveries of BE, which is the most basic metabolite (pK_a value 11 compared to 8.5 for the other metabolites). As a compromise 1.5% (v/v) ammonia in the eluting solvent was the least that could be used for good recovery values.

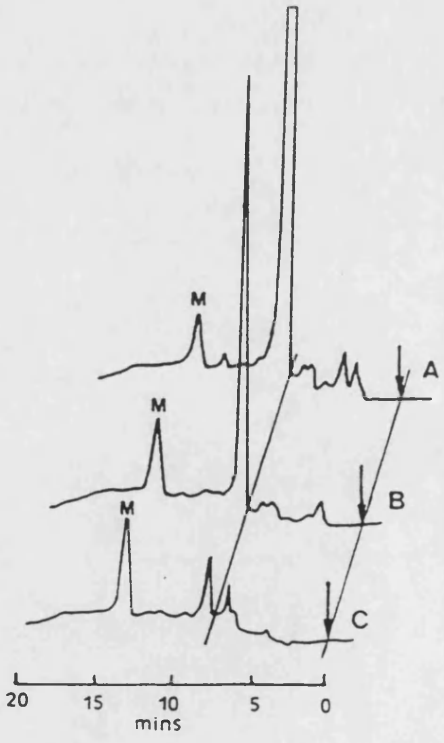

Figure 2
Effect of the percentage ammonia present in the dichloromethane–isopropyl alcohol elution step on Bond-Elut 'Certify' cartridges. No standards or urine was added to the cartridges. Methadone was added after the elution step as a marker. The cartridge residues were derivatized and alkylated as for the cocaine analysis. A = 2% ammonia, B = 1% ammonia, and C = 0.5% ammonia.

In this application the derivatization procedures to increase the hydrophobicity of BE, NC and EME have two major benefits. Firstly their chromatographic retention times are increased, which moves them further away from the endogenous peaks extracted from urine, and secondly it improves the extraction of the ion-pair complexes into the organic phase and so increases sensitivity.

For benzoylecgonine and norcocaine, alkylation is appropriate but not the ethyl derivatives because cocaine is metabolized to ethylcocaine by those persons who also abuse alcohol. Initially the procedure of Ortuno *et al.* [11] was used with iodobutane replacing iodoethane. Derivatization was satisfactory and reproducible, but required 3 h at 60°C. Since crown ethers have been used as catalysts for alkyl halide derivatizations [15], 18 Crown 6 ether was selected in this study to speed up the reaction. Acetonitrile was used as the solvent instead of acetone so that the reaction temperature could be raised to 85°C and the vial caps remain secure. Acetonitrile is also more compatible with the silylation step.

18 Crown 6 ether is soluble in dichloroethane and produces a large peak near the solvent front, and so to minimize extraction into the post-column system a minimum amount of crown ether was used. Decreasing the amount of crown ether from 2.0 to 0.03 mg ml^{-1} did not make any difference to the derivatization yield or time required.

Silylation is widely used in GC to form trialkylsilyl ethers for compounds which contain hydroxyl groups. Most of the silylating agents are very sensitive to moisture and cannot be used for RP-HPLC. Tertiary butyldimethylsiloxyl groups (TBDMS) are 10^4 times more stable than tertiary methyl siloxyl groups [16, 17]. MTBSTFA was found to form a stable *t*-butyldimethlsilyl ecgonine methyl ester derivative which is resistant to hydrolysis for more than 1 week at room temperature. It is important to exclude any moisture during the derivatization procedure in order to obtain a successful, and reproducible derivatization.

The Supelco pKb 100 column is marketed for the chromatography of basic compounds and gave symmetrical narrow peaks for all the metabolites in this application. The column has been used for over a year without any major deterioration in its performance. A 2.1 mm i.d. column was preferred for this application in order to increase sensitivity and reduce solvent consumption and with care can be used under gradient conditions with little background noise.

Without derivatization, norcocaine appeared just after cocaine and these compounds were difficult to separate under gradient conditions. Changing the mobile phase pH or the organic modifier did not improve the situation. A step gradient was used to shorten the analysis time. Figure 3 shows a chromatogram of a mixture of derivatized standards. Norcocaine as its butyl derivative is well separated from cocaine and appears after ethylcocaine. Norcocaine was not used in spiked urine samples as there was some interferences from the cartridges at its retention time. Fortunately, norcocaine is a very minor metabolite in humans.

In the present application methadone has been used as an internal standard. However, methadone can also be present in the urine of some cocaine users as it is prescribed for the treatment of cocaine addicts. In that case hexylbenzoylecgonine can be used as an internal standard and the method can then also be used to measure methadone (Fig. 3).

The calibration curves for all the standards spiked into urine were linear from 200 to 1000 ng ml^{-1} with correlation coefficients >0.99 (Fig. 4). The equations of the calibrations with standard deviations shown in brackets were as follows: for cocaine, $y = 0.913\ (\pm 0.124)x + 0.011\ (\pm 0.081)$, $n = 4$, $r = 0.999$, limit of detection (LOD) = 0.059 μg ml^{-1}; for ethyl-cocaine, $y = 2.68\ (\pm 0.712)x + 0.097\ (\pm 0.395)$, $n = 4$, $r = 0.995$, LOD = 0.095 μg ml^{-1}; for benzoylecgonine, $y = 1.61\ (\pm 0.165)x - 0.126\ (\pm 0.114)$, $n = 4$, $r = 0.999$, LOD = 0.046 μg ml^{-1}; for ecgonine methyl ester, $y = 0.983\ (\pm 0.156)x - 0.021\ (\pm 0.123)$, $n = 4$, $r = 0.998$, LOD = 0.081 μg ml^{-1}. Within-day precision ($n = 4$) for cocaine was 0.78 ± 0.056, recovery 100%; for ethylcocaine 0.96 ± 0.047, recovery 80%; for benzoylecgonine 0.96 ± 0.045, recovery 91%; for ecgonine methyl ester 0.565 ± 0.037, recovery 98%. Much lower limits of detection are possible if the interfering peaks from the cartridges can be eliminated. The problem of interfering peaks was also observed from the

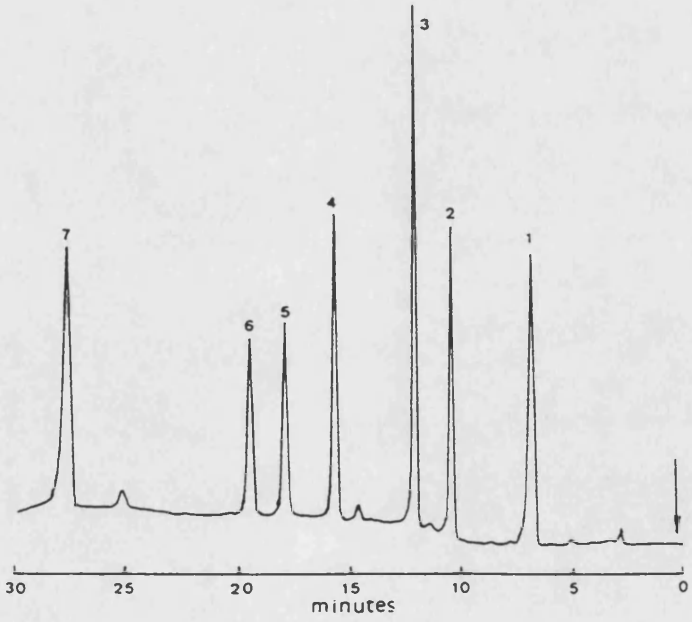

Figure 3
Chromatogram of standards derivatized at 65°C for 3 h [ref. 11] dissolved in mobile phase. 1 = cocaine 0.6 μg ml^{-1}; 2 = ethylcocaine 0.44 μg ml^{-1}; 3 = *N*-butylnorcocaine 0.4 μg ml^{-1}; 4 = butylbenzoylecgonine 0.5 μg ml^{-1}; 5 = methadone 0.3 μg ml^{-1}; 6 = *t*-butyldimethylsilyl ecgonine methyl ester 0.6 μg ml^{-1}; and 7 = hexylbenzoylecgonine 2 μg ml^{-1}. Chromatographic conditions as in Experimental.

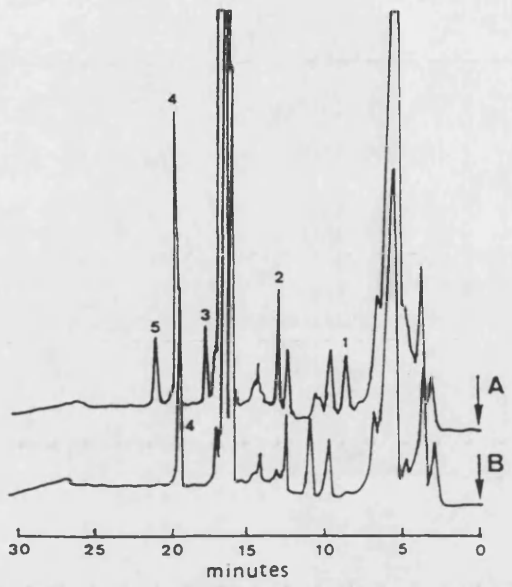

Figure 4
Chromatogram of standards in urine derivatized at 85°C for 1 h in the presence of 18 Crown 6 ether, as in Experimental. A = cocaine and metabolites in urine; B = urine with internal standard. 1 = cocaine 0.23 μg ml^{-1}; 2 = ethylcocaine 0.16 μg ml^{-1}; 3 = butylbenzoylecgonine 0.20 μg ml^{-1}; 4 = methadone 0.75 μg ml^{-1} (internal standard); 5 = *t*-butyldimethylsilyl ecgonine methyl ester 0.2 μg ml^{-1}. Chromatographic conditions as in Experimental.

cartridges of other manufacturers, namely J.T. Baker 'Narc 2' and World-wide Monitoring 'Clean Screen DAU'. This is because the cartridge materials and procedures for cocaine and metabolites recommended by these manufacturers are similar. If the interfering peaks are due to the reaction of ammonia on the silica of the packing material then a cartridge using a polymeric material would be a better alternative. This is currently under investigation.

Throughout the entire study the phase separator was operated at a separation efficiency of 0.8–0.9 without any problems and did not require any major adjustments apart from routine maintenance such as cleaning the needle valve outlet tubings with acetone and blowing air through them.

Conclusions

The method described permits the simultaneous measurement of cocaine, its major metabolites EME and BE, and also ethylcocaine in urine. The method could additionally measure methadone by using hexylbenzoylecgonine as internal standard. The recovery of EME from urine has been increased from typically 40% to at least 80% by the use of larger capacity cartridges. The silyl reagent MTBSTFA produced a stable *t*-butyldimethylsilyl-EME derivative for this LC application. Low pressure gradient systems can be used at low flow-rates, such as 0.4 ml min^{-1}, with 2 mm i.d. columns, provided that the internal volume of the gradient system is reduced.

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PUBLICATION 53

Synthesis of a Deuterated Isotopomer of the Anticancer Drug Hexamethylmelamine and Kinetic Isotope Effect During Biomimetic Oxidation

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Synthesis of a deuterated isotopomer of the anticancer drug hexamethylmelamine and kinetic isotope effect during biomimetic oxidation

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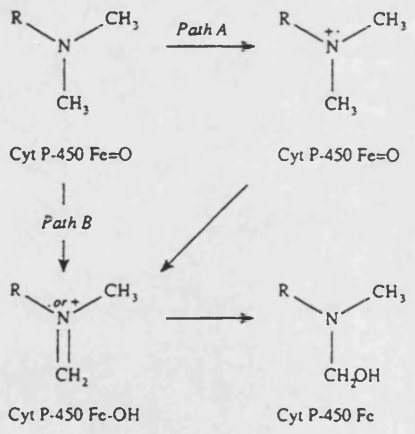
Abstract

Biomimetic oxidation of D_3-hexamethylmelamine (*N,N,N',N',N''*-pentamethyl-*N''*-(trideuteromethyl)-1,3,5-triazine-2,4,6-triamine), an isotopomer of the antineoplastic drug HMM, with chloro-5,10,15,20-tetraphenylporphyrinato-iron(III) / iodosobenzene gives pentamethylmelamine with primary deuterium kinetic isotope effect k_H / k_D = 3.2 ± 0.4. A mechanism involving transfer of H· or H⁻ in the rate-determining step is proposed.

1. INTRODUCTION

Hexamethylmelamine (HMM; **1**, Scheme) is a drug with activity against ovarian cancer. Metabolic hydroxylation, catalysed by a cytochrome P-450, of two or more *N*-methyl groups in the molecule is required for this chemotherapeutic activity [1]. Pentamethylmelamine (PMM; **3**, Scheme 2) is a major metabolite resulting from degradation of an intermediate *N*-CH_2OH compound. The mechanism of this oxidative process is unknown but the mechanisms of metabolic oxidation of several other *N*-methylalkylamines and *N*-methylarylamines by P-450 have been studied [2,3]. Primary kinetic deuterium isotope effects have been used [4] to indicate whether the rate-determining step is transfer of an electron (Path A in Scheme 1) or transfer of hydrogen (as H· or as H⁻) (Path B). Tetraarylporphyrinato-iron (III) / iodosobenzene systems are good models for oxidation by cytochromes P-450 [5], both in terms of chemoselectivity and mechanism of oxidation. As part of a programme of study of metabolism at carbon *alpha* to nitrogen [6,7], the oxidative demethylation of HMM (**1**) by such a model system was selected for investigation.



Scheme 1. Alternative rate-determining steps for hydroxylation at *N*-CH_3 by Cyt. P-450.

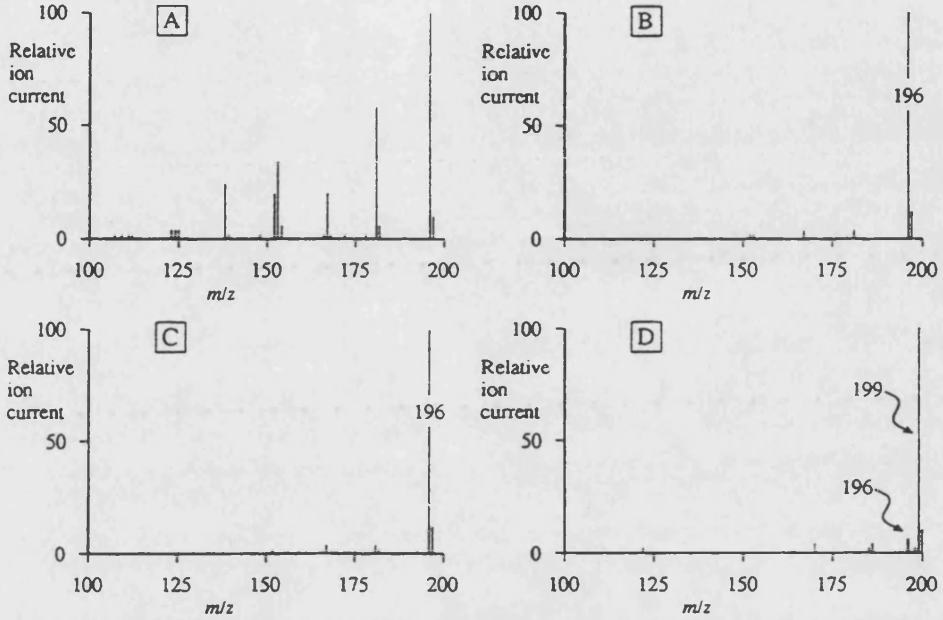


Figure 1. A: E.I. mass spectrum of synthetic unlabelled PMM (3) at 70 eV ionisation energy; B: E.I. m.s. of synthetic unlabelled PMM (3) at lower ionisation energy; C: Low ionisation energy E.I. m.s. of PMM (3) formed from unlabelled HMM (1) by oxidation with TPPFeCl / PhIO; D: Low ionisation energy E.I. m.s. of mixture of isotopomers of PMM (3 + 4) formed by oxidation of D_3-HMM (2) with FeTPPCl / PhIO.

The ratio of isotopomers of PMM (3 and 4) in the product was measured by low ionisation energy E.I. m.s.; a spectrum from a typical run is shown in Figure 1D. The ratio of 3 to 4 in the product mixture was determined from the ratio of abundance of molecular ions at *m/z* 196 and *m/z* 199, respectively. After correction for statistical factors (principally the 5:1 ratio of CH_3 to CD_3 in 2), the intramolecular primary kinetic deuterium isotope effect was calculated to be k_H / k_D = 3.2 ± 0.4 (n = 5 experiments).

4. CONCLUSIONS

This value of intramolecular kinetic deuterium isotope effect (k_H / k_D = 3.2) is consistent with a mechanism of oxidation involving cleavage of the carbon-hydrogen bond in the *N*-methyl group during the rate-determining step in the formation of the corresponding *N*-(hydroxymethyl) moiety. This latter compound is unstable under the reaction conditions and decomposes to the *N*-H derivative (PMM) in a step not involving cleavage of C-H or C-D bonds. Thus, the biomimetic oxidative process is suggested to proceed *via* initial transfer of H· or H· from the substrate to the intermediate oxoporphyrinato-iron, in analogy with the proposed mechanisms of oxidation of the

2. CHEMICAL SYNTHESIS

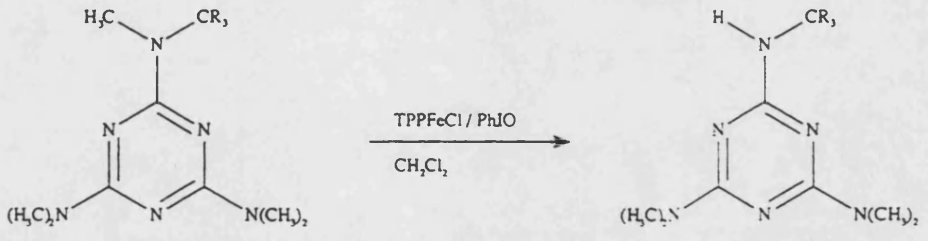
Unlabelled hexamethylmelamine (1) was prepared from 2,4,6-trichloro-1,3,5-triazine by treatment with boiling aqueous dimethylamine; unlabelled pentamethylmelamine (3) was prepared from 6-chloro-*N,N,N',N'*-tetramethyl-1,3,5-triazine-2,4-diamine, according to the method of Borkovec and DeMilo [8]. The trideutero isotopomer 2 was synthesised in 75% yield by *(i)* treatment of PMM (3) with *n*-butyl lithium and *(ii)* reaction of the anion with trideuteroiodomethane, as previously described [9]. Isotopic purity was >99% as judged by mass spectrometry. Chlorination of iodobenzene and subsequent hydrolysis of the intermediate $PhICl_2$ with aqueous NaOH furnished the oxidant, iodosobenzene [10].

3. BIOMIMETIC OXIDATION

To minimise artifacts from isotope effects on fragmentation in the mass spectrometer, spectra of synthetic unlabelled PMM (3) were obtained under various spectroscopic conditions. Electron impact (E.I.) mass spectra using the standard ionisation energy (70 eV) contained many peaks due to daughter ions (Figure 1A); however, lowering the ionisation energy gave spectra exemplified by Figure 1B, in which the molecular ions (*m/z* 196 ($^{12}C_9H_{18}N_6$) and *m/z* 197 ($^{12}C_8{}^{13}C_1H_{18}N_6$)) account for >95% of the total ion current.

Treatment of unlabelled hexamethylmelamine (1) with iodosobenzene (PhIO) and a catalytic amount of chloro-tetraphenylporphyrinato-iron (III) (TPPFeCl) in CH_2Cl_2 gave pentamethylmelamine (PMM; 3) which was characterised by comparison with synthetic material using thin layer chromatography (silica gel, various solvents) and electron-impact mass spectrometry. An example of a mass spectrum of PMM formed by such an oxidative demethylation is shown in Figure 1C. The rate of reaction was found to be sensitive to the state of division of the sparingly soluble PhIO and all quantitative experiments were performed using one batch of finely powdered material; the substrate and metal complex were soluble. Addition of large excesses of PhIO caused destruction of the catalyst.

For the measurement of the isotope effect, D_3-HMM (2) was treated with TPPFeCl and PhIO (*ca.* 15 mol%) so as to allow conversion of 10% of the substrate, thus artifacts due to selective depletion of the substrate and due to consumption of the product are minimised.



1: R = H
2: R = D

3: R = H
4: R = D

Scheme 2. Biomimetic oxidation of isotopomers of hexamethylmelamine.

electron-poor *N*-methylamides [11] and formamides [7] by cytochrome P-450 but in contrast to the initial transfer of an electron from the relatively electron-rich *N*-methylanilines and *N*-methylalkylamines to the cytochrome [3].

5. EXPERIMENTAL

5.1. Chemical synthesis

D_3-HMM (*N,N,N',N',N''*-pentamethyl-*N''*-(trideuteromethyl)-1,3,5-triazine-2,4,6-triamine; 2) was synthesised in 75% yield and 99% isotopic purity by alkylation of the lithium salt of pentamethylmelamine (*N,N,N',N',N''*-pentamethyl-1,3,5-triazine-2,4,6-triamine; 3 [8]) with CD_3I (Aldrich Chemical Co., Gillingham, U.K.) in dry THF, as previously described [9].

5.2. Biomimetic oxidation / kinetic isotope effect

Typical experiment: D_3-HMM (2) (40.0 mg, 0.188 mmol) was dissolved in CH_2Cl_2 (2.5 ml) and chloro-tetraphenylporphyrinato-iron (III) (14.0 mg, 0.020 mmol) was added, followed by iodosobenzene (6.6 mg, 0.030 mmol). The mixture was stirred for 30 min at ambient temperature. The mixture of isotopomers of pentamethylmelamine (3 + 4) was isolated from the evaporation residue by centrifugally accelerated preparative layer chromatography (silica gel, chloroform/methanol 19:1; twice), giving off-white crystals (3.8 mg, 10% based on 2). The ratio of isotopomers was determined by mass spectrometry using a VG 7070 instrument in the E.I. mode.

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Relationship between the Melanin Content of a Human Melanoma Cell Line, its Radiosensitivity and Uptake of Pimonidazole

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Relationship between the melanin content of a human melanoma cell line and its radiosensitivity and uptake of pimonidazole*

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Summary. The intra-cellular uptake of the weakly basic radiosensitiser pimonidazole (PIMO) was determined as a function of the pigmentation of Nal1+ human melanotic melanoma cells in vitro. Two experimental conditions were considered: exponentially growing cells (Exp.) and plateau-phase cells (Pl.). The melanin content of Nal1+ cells ranged from 500 μg/g cell weight in exponentially growing cells to 6000 μg/g in heavily pigmented plateau-phase cells. Cells were exposed to PIMO (medium dose, 0.2 mmol/dm^3; 58.2 μg/ml). The intra-cellular concentration ranged from 163 μg/g in Exp. to 900 μg/g in pigmented Pl.; the latter being equivalent to an intra- to extracellular concentration ratio (Ci/Ce) of 17. However, this increase in the cellular uptake of PIMO was not accompanied by an increase in radiosensitising efficiency. In comparison, the Ci/Ce for etanidazole (ETA), a radiosensitiser that is uncharged at physiological pH, remained approximately constant at 1 for all values of melanin contents. Treatment of Nal1+ tumours in vivo with [^{3}H]-PIMO resulted in a tumour: blood ratio of about 3 at 30–60 min after administration. However, at 24 h a grain count of label derived from [^{3}H]-PIMO showed that picnotic areas of tumours contained levels that were some 40 times greater than the background value. This high level of label was coincident with areas of highest apparent melanin content. In conclusion, PIMO accumulates in very heavily pigmented melanoma cells present in necrotic zones with picnosis. As these cells are probably non-clonogenic, PIMO is not suitable for use in melanoma radiotherapy.

Introduction

Most rodent and human xenografted tumours contain hypoxic cells [12, 25, 28], and a few clinical studies have suggested that radiotherapy might be improved by the use of agents such as nitroimidazoles that increase the radiosensitivity of these hypoxic cells [6]. The first agents evaluated were metronidazole and misonidazole, but neurotoxicity has limited their use in radiotherapy. A second generation of hypoxic cell sensitisers have been developed, among which pimonidazole (PIMO) is particularly interesting since it is preferentially accumulated by tumour cells in vitro [4]. PIMO also accumulates in rodent tumours as indicated by tumour: blood ratios of >1 [15, 20, 21, 24, 33, 37, 44–46] and, similarly, in human tumour xenografts [21, 35] and in human tumours in patients [1, 8, 26], with the uptake being highest in melanomas as compared with other tumours [1, 5, 8, 20, 21, 26]. The high tumour-cell concentration of PIMO that can be obtained suggests that the radiosensitising effect of PIMO should also be high, and the results obtained using non-melanotic cells growing exponentially in vitro support this hypothesis [30, 40]. In addition, PIMO has generally been found to be effective in non-pigmented rodent tumours in vivo [15, 16, 32, 44]. However, using human tumour xenografts and clinically relevant drug doses, a radiosensitising effect has been detected in rectal adenocarcinoma HRT18 but not in melanoma Nal1+, although the accumulation of PIMO in the melanoma was particularly high [21, 35]. We report the results of some studies that may explain the basis for the lack of effect of PIMO on the Nal1+ melanoma.

Tumours are very heterogeneous, especially with respect to the cell-proliferation kinetics (exponentially growing cells, plateau-phase cells) and to the presence of necrotic zones. This heterogeneity suggests that the average intra-tumour concentration may not necessarily reflect the concentration of the drug in the clonogenic hypoxic cells, which are the cells essential for tumour radiosensitivity. In vitro radiosensitisation and drug uptake have previously been investigated in exponentially growing cells [30, 38–41]. Therefore, the aim of the present work was, firstly, to measure the uptake of PIMO into exponentially growing and plateau-phase melanotic melanoma cells in vitro and determine whether this might be related to their melanin content and secondly, to determine whether the

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inter- and intra-cellular distribution of [^{3}H]-PIMO in Na11+ tumours might explain the lack of radiosensitisation observed for PIMO in this tumour type.

Materials and methods

Compounds. Etanidazole [ETA, *N*-(2-hydroxyethyl)-2-nitroimidazolyl acetamide: Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, USA] and pimonidazole {PIMO, α-[(2-nitro-1-imidazolyl)methyl]-1-piperidine-ethanol hydrochloride: Hoffman-La Roche, Switzerland} were dissolved in minimum essential medium (MEM) supplemented with 20 mM HEPES (pH 7.2) at a concentration of 0.2 mmol/dm^3 (42.8 μg/ml for ETA and 58.2 μg/ml for PIMO). [^{3}H]-PIMO [42] dissolved in absolute ethanol had a specific activity of 5.5×10^7 Bq/ml. [^{3}H]-PIMO (10 μl for scintillation or 100 μl for autoradiography) was mixed with non-radioactive PIMO dissolved in Dulbecco's phosphate-buffered saline (PBS). The total amount of PIMO injected i. v. into mice via the retro-orbital sinus in a volume of 0.3 ml was 200 mg/g body weight. Control mice were given PBS alone.

Tumour-cell system. The melanotic melanoma Na11+ originated from a human melanoma. The characteristics and maintenance of this cell line have been described elsewhere [14]. Congenitally athymic nude mice were bred and maintained in a defined flora- and pathogen-free colony. Details of the mouse breeding and tumour production have been published elsewhere [13, 14]. Tumours were obtained by injecting 3×10^6 cells into both flanks of mice that had been whole-body-irradiated with 5 Gy ^{137}Cs γ-rays to increase tumour uptake.

In vitro studies. Plateau-phase cells were obtained 7 days after 5×10^5 cells had been seeded in glass petri dishes (unpublished data). A few experiments were performed using exponentially growing cells obtained 2 days after the cells had been seeded. For the aerobic study, cells were placed in an incubator (37° C, 5% CO_2, 45 min). For hypoxia, open dishes were placed in aluminium chambers and gassed with a humidified mixture of 95% N_2 and 5% CO_2 (<3 ppm O_2) for 45 min. A filtered solution of dithionite-sodium carbonate was placed in the center of the chambers to remove traces of oxygen [18]. After undergoing incubation with PIMO or ETA and/or irradiation, the cells were trypsinised and the surviving fraction was assessed by an in vitro colony assay. Colonies were fixed and stained with crystal violet (0.25%, w/v, in 80% methanol containing 10% formaldehyde).

Cellular uptake and drug concentration in the medium was measured by high-performance liquid chromatography (HPLC) [21, 23]. Briefly, the medium was analysed directly and cells were collected with a rubber policeman and stored at –80° C. On the day of analysis, the cells were sonicated in water and ETA and PIMO were extracted with acetonitrile-water (1 : 1, v/v). The extraction was repeated and the supernatants were pooled and evaporated to dryness. The residue was diluted in eluent B containing an appropriate amount of internal standard (Ro 03-1902), and cellular uptake was analysed using a Varian model 5000 chromatograph equipped with a 5-μm Nucleosil column connected to a Varichrom UV-visible detector at 326 nm. The elution flow rate was 2 ml/min (eluent A, 75% acetonitrile and 25% water; eluent B, 4 mM heptane sulphonic acid, 5 mM dibutylamine and 50 mM glycine adjusted to pH 3); the gradient was: 0 min, 8% eluent A; 10 min, 45% eluent A; 10–11 min, 45%–8% eluent A.

In vivo studies. Tumours were used when they had reached a mean diameter of 9–11 mm. Animals were anaesthetised at various times after drug injection and blood was collected by cardiac puncture into heparinised tubes immediately before tumour excision and frozen in liquid nitrogen. Tumours were removed and immediately frozen in liquid nitrogen. Subsequently, tissue samples were weighed and 100–300 mg was suspended in Optisolve (LKB). Tissues were digested by incubation at 55° C for 16 h. 15 ml liquid scintillation fluid (Optiphase, Hisafe-TM; LKB) was added and samples were counted on a Packard Tricard liquid scintillation counter. A set of variably quenched standards were used for calibration. These were prepared by adding a known activity of tritiated water to a set of eight samples containing between 0 and 0.2 ml whole blood digested as described above. Any very highly coloured samples were bleached by incubation at 60° C for 2 h with benzoyl peroxide [0.4 ml, 5% (w/v) in toluene] prior to the addition of scintillant.

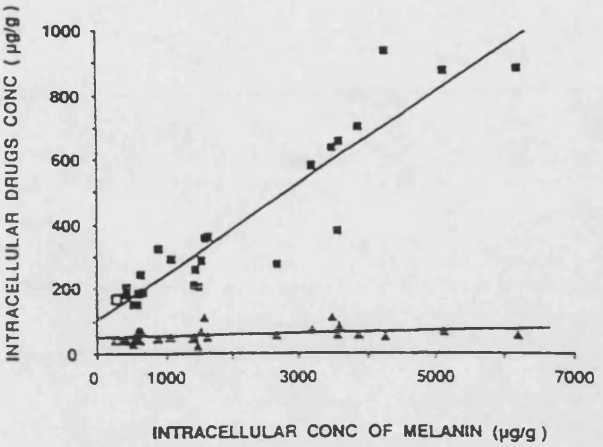


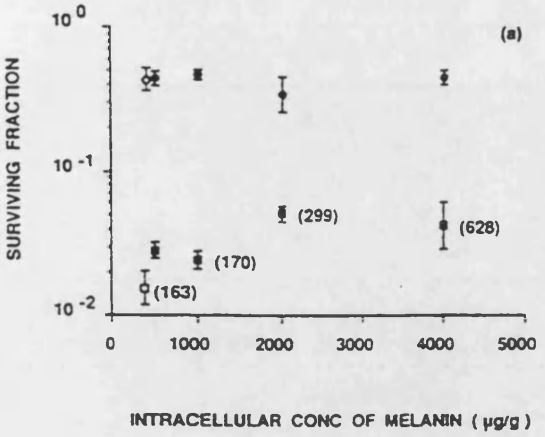
Fig. 1. Intra-cellular concentrations of ETA (▲, △) and PIMO (■, □) as a function of melanin content. *Closed symbols*, plateau-phase cells; *open symbols*, exponentially growing cells

Histology, autoradiography and melanin determination. Tumours were removed at 24 h after [^{3}H]-PIMO administration and were immediately fixed in ethanol: acetic acid (3 : 1, v/v). The tissues were dehydrated and embedded in paraffin and sections (3 μm) were cut and mounted on microscope slides. For autoradiography the slides were dipped in Ilford K2 emulsion and exposed for 2 months. The slides were then developed in Kodak D19b and stained with hemalum and erythrosin. The amount of radioactivity in the tumour was determined by grain counting using an ocular grid. The percentage of cellular areas was identified under a microscope and outlined on photographs of the sections. The areas were weighed and cellular areas were expressed as a percentage of the total areas. Melanin was localized by the method of Fontana-Masson [17].

For melanin determination, the quantitative colourimetric method of Foster et al. [9, 10] as adapted to normal and cultured retinal pigment cells by Whittaker [43] was used, with a few modifications. Cells were sonicated in water, and non-melanic substances that interfered with the assay were removed by three extractions with 5% trichloroacetic acid, two extractions with cold ether-alcohol (1 : 3, v/v) and one extraction with absolute ether. The dried residue extracted from 50–100 mg cells was dissolved in 1 ml 1 *N* KOH and then heated to 100° C for 30 min. It was cooled to room temperature and the optical density was measured at 400 nm (Varian spectrophotometer). A standard curve was constructed using synthetic melanin (Sigma) dissolved in 1 *N* KOH (1–100 μg/ml). Absorbance at 400 nm increased linearly with melanin concentration up to 100 μg/ml.

Results

A preliminary experiment showed that the melanin content was very homogeneous in exponentially growing cells but varied greatly in plateau-phase cells. The melanin content of plateau-phase cells obtained at 7 days after seeding increased by a factor of >6 within 12 h (results not shown). The uptake of PIMO into hypoxic plateau-phase Na11+ cells in vitro was compared with that of ETA. Figure 1 shows that the intra-cellular concentration of PIMO increased linearly (correlation coefficient, 0.9 P:10^{-4}) as a



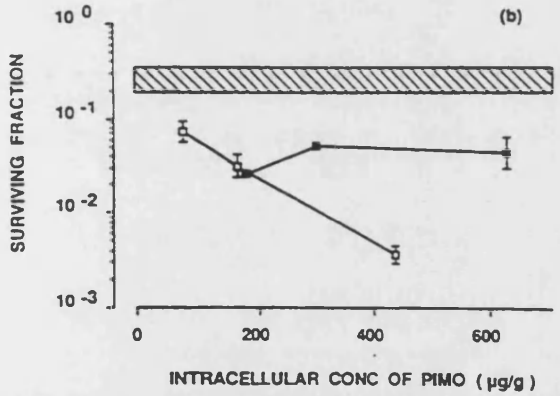


Fig. 2 a, b. Surviving fractions of Na11+ Exp. (*open symbols*) and Na11+ Pl. (*closed symbols*) after the delivery of a radiation dose of 10 Gy under hypoxic conditions in the presence or absence of PIMO. a As a function of intra-cellular melanin concentration: (○, ●), in the absence of PIMO; (□, ■), in the presence of PIMO. The intra-cellular PIMO concentration is shown (in µg/g of cells) *in parentheses*. b As a function of intra-cellular PIMO concentration. The *shaded area* represents the 95% confidence interval of cell survival in nitrogen without PIMO. Data points represent mean values for 2 experiments: error bars indicate 95% confidence intervals

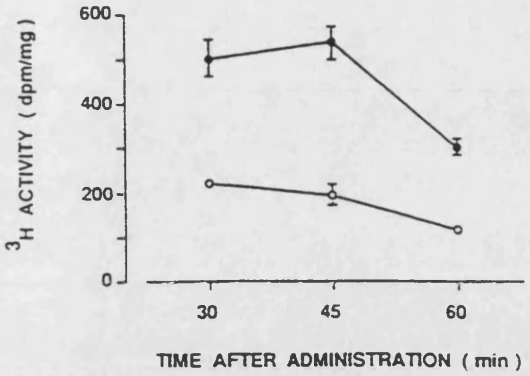


Fig. 3. ^{3}H activity in blood (○) and tumours (●) after the injection of [^{3}H]-PIMO. Data represent mean values ± 95% confidence intervals

function of melanin content. At the lowest melanin content (500 µg/g), the Ci/Ce ratio for PIMO was between 3 and 4, which is very similar to that found for exponentially growing Na11+ cells. However, in plateau-phase cells in which the melanin content increased, the Ci/Ce value also increased to >17. In contrast, the intra-cellular concentration of ETA remained fairly constant, with Ci/Ce values being close to 1.

The radiosensitising effect of 58.2 µg PIMO/ml in hypoxic Na11+ cells was determined as a function of cellular pigmentation. Figure 2 shows that there was no significant change in the radiosensitivity with pigmentation in plateau-phase cells following a radiation dose of 10 Gy in the presence of PIMO (Fig. 2a), although the intra-cellular PIMO concentration measured in the more pigmented cells was at least 3-fold that determined in the less pigmented cells (Fig. 1). In these experiments, no significant change in the plating efficiency was detected as a function of either the pigmentation or the intra-cellular PIMO concentration. In Fig. 2b, these results expressed as intra-cellular concentration (Ci) are compared with those obtained for radiosensitisation of exponentially growing cells (melanin content, <500 µg/g). The extra-cellular concentration (Ce) remained constant (58.2 µg/ml) in plateau-phase cells, whereas it varied from 29.1 to 174.6 µg/g in exponentially growing cells. Clearly, in exponential Na11+ cells, radiosensitisation by PIMO increases as a function of Ci. In contrast, no similar increase in radiosensitisation was observed in plateau-phase cells. Furthermore, the maximal Ci value obtained in exponential cells (437 µg/g) was limited by toxicity, whereas in plateau-phase cells a Ci value of 628 µg/g was achieved with no loss in plating efficiency.

Liquid scintillation studies

[^{3}H]-PIMO radioactivity in the blood and in the tumour is shown in Fig. 3. The radioactivity in the blood decreased with time after administration, whereas that in the tumour increased to a plateau. The tumour/blood ratio was relatively constant (2–3) at between 30 and 60 min.

Histology studies

The cellular zone represented only 51% of the tumour. Figure 4 shows the melanin content, with the highest concentrations of melanin being found in the zones of tumour in which picnotic cells form the largest proportion of cells (Fig. 4b). The radioactivity derived from [^{3}H]-PIMO was not evenly distributed throughout the tumour. The results of grain counts from two experiments are given in Table 1: it is apparent that the necrotic zones containing numerous picnoses were most highly labeled, showing values some 40 times greater than the background level. In contrast, viable zones of tumour and necrotic regions containing no identifiable cell fragments or picnotic cells showed substantially lower grain counts. Only one-fifth of the total radioactivity was located in cellular zones. Although the

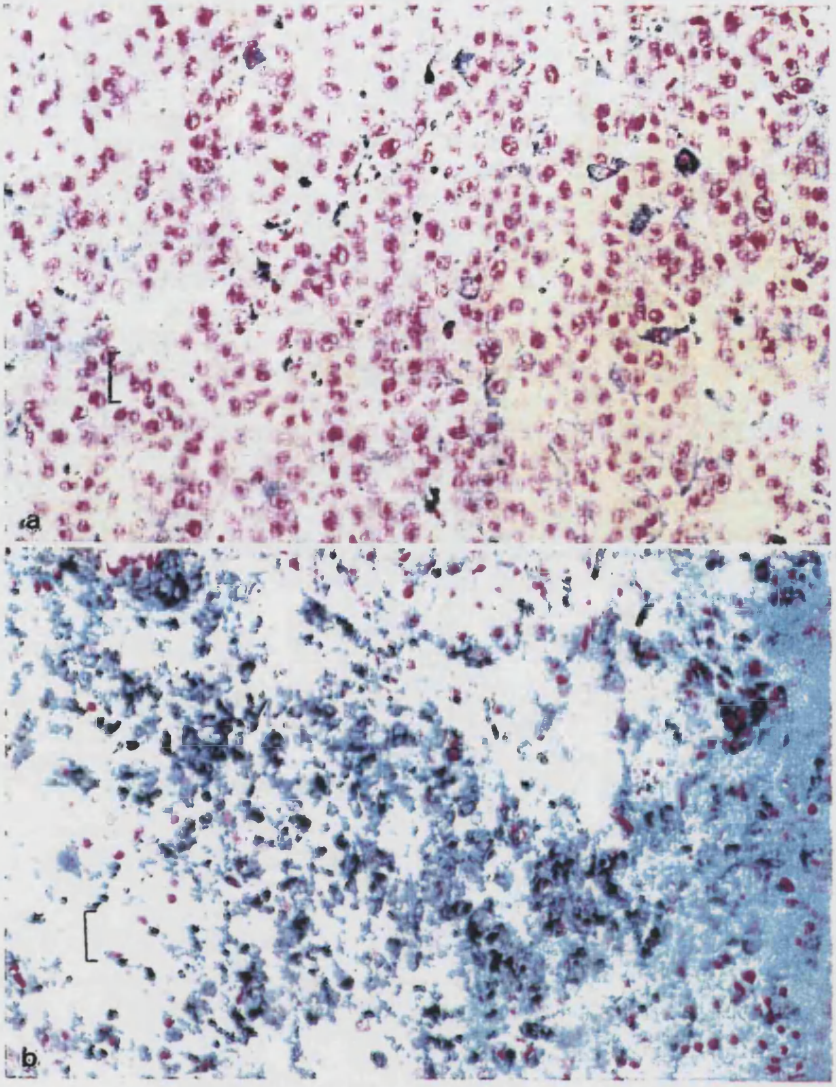


Fig. 4 a, b. Histological sections of Na11+ tumours showing melanin **a** in the cellular zones and **b** in necrotic zones where picnotic cells are found. Bar = 40 µm

Table 1. Distribution of radioactivity derived from [^{3}H]-PIMO in tumours and percentage of total radioactivity

	Tumour 1			Tumour 2		
	Cellular zone (CZ)[a]	Necrotic zone		Cellular zone (CZ)[a]	Necrotic zone	
		With picnotic cells and cell fragments (NZP)[b]	Without any tumour-cell material (NZ)		With picnotic cells and cell fragments (NZP)[b]	Without any tumour-cell material (NZ)
Mean number of grains (MNG)	156 (20)	621 (86)	209 (21)	140 (7)	533 (30)	81 (13)
% Total area (TA)	52	35	13	44	43	13
% Total radioactivity[c]	25	67	8	20	76	4

Numbers in parentheses represent standard errors; 8–40 fields were studied per zone (background value = 15)

[a] See Fig. 4 a for the histology of the cellular zone

[b] See Fig. 4 b for the histology of the necrotic zone with picnotic cells and cell fragments

[c] % Total radioactivity: $MNG_{CZ} \times \%\ TA_{CZ} + MNG_{NZP} \times \%\ TA_{NZP} + MNG_{NZ} \times \%\ TA_{NZ} = 100\%$

melanin content and the radioactivity derived from [^{3}H]-PIMO were not identified on the same slide, it is clear that the highest activity and the highest staining of melanin were found in the same zones.

Discussion

It has been suggested that weak bases can concentrate in melanin-containing cells [3, 20–22, 31, 35, 36]. The intracellular concentrations of weak bases such as PIMO [4], RSU 1069 and RSU 1164 [37] are increased at elevated extra-cellular pH, and their much higher uptake into melanotic as compared with non-melanotic cells led to the suggestion that the intra-cellular pH of melanotic melanoma cells is lower than the pH of non-pigmented cells. However, other mechanisms are likely to be involved in the concentration of weak bases in melanotic melanomas. The present in vitro results show that the pigmentation is higher in plateau-phase cells relative to exponentially growing Nal 1+ cells; furthermore, when the cells reach the plateau phase, a continuous and rapid increase in pigmentation is observed. For plateau-phase melanotic cells, the results clearly show that the intra-cellular concentration of PIMO depends strongly on the intra-cellular concentration of melanin: the higher the melanin content, the higher the PIMO concentration (Fig. 1). PIMO had no greater radiosensitising effect on these cells than it had on exponentially growing cells, despite the observation that the intra-cellular concentration of PIMO was higher and the plating efficiency was not modified. An association between PIMO and melanin could explain these results, especially if the melanin is remote from the DNA [34], thus spatially preventing PIMO from exerting its radiosensitising effect.

The data for melanoma transplanted into nude mice show that at a short time after its administration, PIMO accumulates in tumours (Fig. 3). Histological data derived from tumours excised at 24 h after the administration of [^{3}H]-PIMO show the presence of label to be coincident with areas of high melanin content, which would be consistent with the in vitro results. It is unlikely that this localisation would be a consequence of passive sequestration onto or association with melanin; rather, it is probably due to selective hypoxia-induced binding in these areas. In non-melanotic tumours it has been claimed that [^{14}C]-misonidazole [2, 11] and PIMO [19, 29] accumulate in viable hypoxic tumour cells, whereas other investigators have reported that uptake is lower in necrotic tumours [7, 27] or that there is no link between accumulation and necrosis [21, 26]. On the basis of our results, it is difficult to determine the influence of the necrotic areas on PIMO accumulation, as these regions were also those in which the melanin content was the highest. It must be emphasised that although it was not possible to evaluate the melanin content in the necrotic zones, these dying cells probably contained much more melanin than did the plateau-phase cells we studied; indeed, the plateau obtained in the present studies is not a perfect one, since 11% of the cells remained in the S phase (unpublished results).

On the basis of the above-mentioned observations, it is possible to provide an explanation for our previous results, which indicated an inability of PIMO to sensitise Nal 1+ tumours [35]. The accumulation of PIMO in the tumour (liquid scintillation studies) may well have resulted from the accumulation of PIMO in necrotic zones containing picnotic cells of very high melanin content. Its accumulation in these regions cannot influence radiosensitivity; only the PIMO in areas of clonogenic hypoxic cells is important.

In conclusion, the present results obtained both in vitro and in vivo indicate that PIMO accumulates in very heavily pigmented melanoma cells. The in vivo results also show that the accumulation of label derived from PIMO is higher in the necrotic zones with picnosis than in the cellular areas. The latter findings could explain why the intra-cellular accumulation of PIMO is not linked with a radiosensitising effect in melanotic melanoma. To explain the lack of efficiency of PIMO in vitro, a mechanism such as the localisation of PIMO far from the DNA close to the melanin content or a very low sub-cellular pH must be supposed. As far as melanotic melanomas are concerned, PIMO is probably not the compound to be used in clinical radiotherapy.

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PUBLICATION 55

Investigation of the Mechanistic Basis of *N,N*-Dimethylformamide Toxicity. Metabolism of *N,N*-Dimethylformamide and its Deuterated Isotopomers by Cytochrome P450 2E1

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Investigation of the Mechanistic Basis of *N,N*-Dimethylformamide Toxicity. Metabolism of *N,N*-Dimethylformamide and Its Deuterated Isotopomers by Cytochrome P450 2E1

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Dimethylformamide (DMF) is an industrial solvent with hepatotoxic properties. The toxicity of DMF has been associated with its metabolism to *S*-(*N*-methylcarbamoyl)glutathione (SMG). The major urinary metabolite of DMF is *N*-(hydroxymethyl)-*N*-methylformamide (HMMF). HMMF undergoes oxidation in the formyl moiety, possibly via the intermediacy of its hydrolysis product *N*-methylformamide (NMF), and the reactive intermediate thus generated reacts with glutathione to yield SMG. Experiments were conducted to elucidate enzymatic details of the metabolism of DMF. Generation of HMMF from DMF in microsomes from rats which had received acetone, an inducer of cytochrome P450 2E1, was increased by 175% over that observed in control microsomes. In liver microsomes from 4 humans the metabolism of DMF to HMMF was inhibited by a monospecific antibody against rat liver P450 2E1, and the metabolic rates were correlated with those of NMF to SMG, a process known to be mediated via P450 2E1. DMF was also metabolized by purified rat liver P450 2E1. The kinetic parameters which characterize the metabolism of DMF or its deuterated isotopomers to the respective HMMF isotopomers, of HMMF to SMG and of NMF to SMG in liver microsomes, were computed from Eadie–Hofstee plots. The affinity of DMF for the metabolizing enzyme in rat liver microsomes is considerably higher (apparent K_m = 0.20 mM) than that of NMF (K_m = 4.28 mM) or of HMMF (K_m = 2.52 mM). The respective values observed with human microsomes are very similar. The apparent K_m values for the *N*-methyl oxidation of *N,N*-dimethyldeuterioformamide ([$^2H_1$]DMF) and *N,N*-bis(trideuteriomethyl)formamide ([$^2H_6$]DMF) in rat microsomes are 0.14 and 0.21 mM, respectively. The apparent V_{max} for the oxidation of [$^2H_1$]DMF is similar to that computed for DMF, and the V_{max} for [$^2H_6$]DMF is less than half of that computed for DMF. The kinetic deuterium isotope effect (KDIE) on DMF metabolism was determined in incubations with rat microsomes in three ways: (i) the noncompetitive intermolecular KDIE by the ratio of V_{max}/K_m for DMF to V_{max}/K_m for [$^2H_6$]DMF, (ii) the competitive intermolecular KDIE as the quotient of metabolic products HMMF to *N*-(hydroxydideuteriomethyl)-*N*-(trideuteriomethyl)formamide in incubations of DMF together with [$^2H_6$]DMF, and (iii) the intramolecular KDIE as the quotient of the ratio of *N*-(hydroxymethyl)-*N*-(trideuteriomethyl)formamide to *N*-(hydroxydideuteriomethyl)-*N*-methylformamide generated from *N*-(trideuteriomethyl)-*N*-methylformamide ([$^2H_3$]DMF). The respective values were found to be (i) 2.4, (ii) 5.0, and (iii) 5.2. DMF inhibited the oxidation of NMF or HMMF to SMG. Deuterium substitution of the DMF methyl hydrogens did not affect the apparent K_i for the inhibition of the oxidation of NMF to SMG. Among a series of 8 formamides and acetamides structurally related to DMF only *N,N*-diethylformamide and *N,N*-dimethylacetamide were equally effective with DMF as inhibitors. The results suggest that (i) hepatic P450 2E1 is an important catalyst of the metabolism of DMF and related low-molecular-weight amides, (ii) DMF inhibits its own metabolic toxification, and (iii) there is a marked KDIE on the metabolic oxidation of DMF.

Introduction

N,N-Dimethylformamide [DMF,[1] $(CH_3)_2NCHO$] is a polar solvent used widely in a variety of industrial processes, among them the manufacture of synthetic fibers, leathers, films, and surface coatings. Worldwide production of DMF annually has been estimated to be 2×10^5 tons (2). Gastric irritation and hepatotoxicity are the major untoward effects which have been reported in workers exposed to DMF (for review see ref 3). For example, an elevation in biochemical indices of hepatotoxicity has been described recently in workers occupationally exposed to DMF under conditions of poor industrial hygiene (4, 5). These reports have been considered of sufficient gravity

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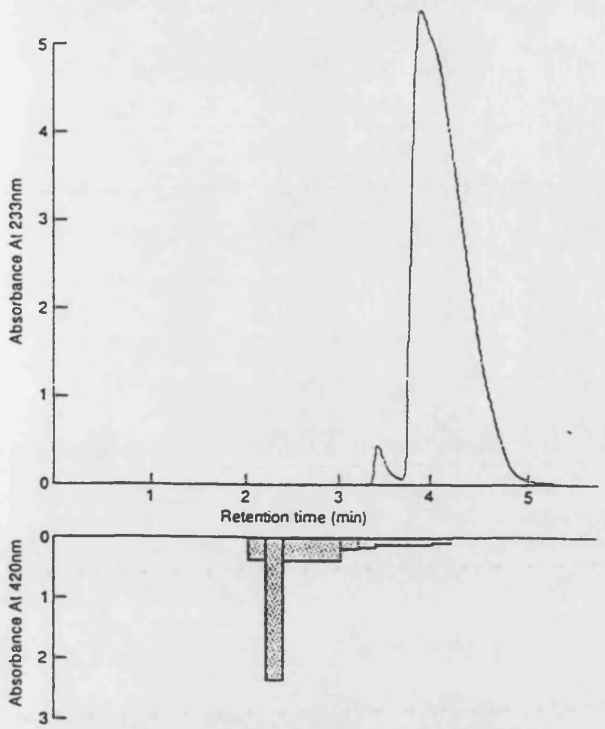

Figure 3. HPLC separation of HMMF (retention time 3.9 min) from NMF (retention time 3.4 min) (top trace) and formaldehyde (bottom trace). Formaldehyde content in the fractions of the HPLC eluate was determined using the colorimetric method of Nash (*24*). Values on the *y*-axis are arbitrary.

Experimental Procedures

Chemicals. DMF, NMF, *N,N*-dimethylformamide, *N,N*-dibutylformamide, *N*-ethylformamide, *N,N*-dimethylacetamide, *N,N*-diethylacetamide, *N*-methylacetamide, formamide, acetamide, and diethyldithiocarbamate (DEDTC) were bought from Aldrich Chemical Co. (Poole, U.K.). DMF and NMF were purified by distillation to >99% purity (GLC) prior to use. [$^2H_6$]-DMF was prepared from [$^2H_6$]dimethylamine hydrochloride (Sigma Chemical Co. Ltd., Poole, U.K.) and ethylformate essentially according to the method described for the synthesis of *N*-(trideuteriomethyl)formamide ([$^2H_3$]NMF) (*22*). In analogy [$^2H_1$]DMF was prepared from dimethylamine hydrochloride and [$^2H_1$]methylformate (Aldrich) and [$^2H_3$]DMF from ethylformate and *N*-(trideuteriomethyl)-*N*-methylamine hydrochloride, which was kindly provided by Dr. R. P. Hanzlik (University of Kansas). The purity of the synthesized isotopomers was verified by GLC and ^{1}H-NMR spectroscopy. Biochemicals (glucose 6-phosphate, glucose-6-phosphate dehydrogenase, GSH, NADP) were obtained from Sigma. HMMF was prepared according to Gate et al. (*23*). The crude product of the synthesis contained 6–8% NMF as determined by HPLC analysis (vide infra) and a similar amount of formaldehyde as determined colorimetrically according to Nash (*24*). The product was purified by semipreparative HPLC to furnish HMMF of suitable purity for in vitro metabolism studies. The HPLC system used for this purpose consisted of a Waters 510 pump, a Waters WISP 710 autoinjector, a Shimadzu SPD-6A UV detector (set at λ = 233 nm), and a Waters Maxima 820 workstation. Separation was achieved on a Lichrospher 100 RP-18 column (250 × 4 mm, 5-μm particle size) through which 10 mM phosphate buffer (pH7.4) was pumped at a flow rate of 1.0 mL/min. Under these conditions the contaminants in 20-μL samples containing 10% HMMF were sufficiently separated from the carbinolamide. Figure 3 demonstrates the separation by HPLC of HMMF, NMF, and formaldehyde in a typical sample of crude product. The HMMF content in the collected eluate was measured by GLC as NMF (vide infra); residual NMF was determined by HPLC as described above. SMG was synthesized by Drs. D. H. Han and P. G. Pearson, University of Washington, Seattle, as described previously (*25*). Propyl *N*-methylcarbamate was prepared from AMCC and 1-propanol using the procedure for derivatization of AMCC in preparation for GLC analysis (*26*). All other chemicals and reagents were available in the laboratory.

Animals and Source of Human Microsomes. Male Sprague-Dawley rats (170–200 g) were purchased from Bantin and Kingmans Ltd. (Hull, U.K.). In some experiments rats were used which had been given acetone as a 50% solution in saline (5 mL kg^{-1}) by single oral gavage 24 h prior to preparation of microsomes (*27*). Control animals received saline. Excess samples of healthy human liver tissue were obtained after graft reduction of donor liver from the Liver Transplant Unit at the Queen Elizabeth Hospital (Birmingham, U.K.). Tissues originated from four organ donors, two females and two males, aged between 10 and 55 years. Homogenate (25%) of human or rodent liver was prepared in Tris-buffered (50 mM, pH 7.4) KCl solution (154 mM). Human liver microsomes, which were prepared within 3 h after liver became available, were stored in liquid nitrogen for up to 12 months before use. P450 2E1 was purified from streptozotocin-treated rats as described previously (*20*).

Preparation of Liver Microsomes and Microsomal Incubations. Microsomes were obtained in the usual way by differential centrifugation of homogenate first at (9×10^3)*g* for 20 min, and then at 10^5*g* for 1 h in a Beckman L8-60M ultracentrifuge. The microsomal pellet was suspended in Tris buffer (50 mM, pH 7.4), recentrifuged at 10^5*g* for 1 h, and resuspended in phosphate buffer (50 mM, pH 7.4). The rat liver microsomes used in each experiment were pooled from 2–4 animals.

All incubations were carried out, with duplicate samples, in 5-mL glass vials under shaking at 37 °C. Incubates contained microsomes (1–2.5 mg of protein·mL^{-1}), a NADPH generating system [glucose 6-phosphate (20 mM), NADP (10 mM), glucose-6-phosphate dehydrogenase (4 IU)], GSH (10 mM), phosphate buffer (50 mM, pH 7.4), and substrate with or without inhibitor at the concentrations decribed below in a final volume of 2 mL. The GSH in the mixture converted metabolically generated *N*-methylcarbamoylating species to SMG (*18*). Reactions were initiated by addition of substrate after a 3-min preincubation period at 37 °C and terminated after incubation for 30 min. Preliminary experiments established that the metabolic generation of HMMF from DMF and that of SMG from NMF or HMMF were linear with time during this time period.

The monospecific antibody against rat liver P450 2E1 was prepared and purified as described recently (*20*).

Preparation of Samples and Quantitation of Metabolites. In preparation for analysis by GLC samples of the incubate were processed as described by Mráz (*26*) and Mráz et al. (*28*). This procedure involves derivatization of metabolically produced SMG with ethanol in alkali to furnish ethyl *N*-methylcarbamate and, at the same time, conversion to HMMF to NMF. Likewise metabolically generated *N*-(hydroxydideuteriomethyl)-*N*-(trideuteriomethyl)deuterioformamide ([$^2H_6$]HMMF) is converted to *N*-(trideuteriomethyl)deuterioformamide ([$^2H_4$]NMF), *N*-(hydroxydideuteriomethyl)-*N*-(trideuteriomethyl)formamide ([$^2H_5$]-HMMF) and *N*-(hydroxymethyl)-*N*-(trideuteriomethyl)formamide ([$^2H_3$]HMMF) to *N*-(trideuteriomethyl)formamide ([$^2H_3$]NMF), *N*-(hydroxydideuteriomethyl)-*N*-methylformamide ([$^2H_2$]HMMF) to NMF, and *N*-(hydroxymethyl)-*N*-methyldeuterioformamide ([$^2H_1$]HMMF) to *N*-methyldeuterioformamide ([$^2H_1$]NMF). Metabolic incubations were terminated by transfer of a sample (1.5 mL) of the incubate into a tube containing ethanol (3 mL) with propyl *N*-methylcarbamate (10 μM), which served as internal standard. For the determination of ethyl *N*-methylcarbamate (the analytical derivative of SMG) an additional extraction step with ethyl acetate was included (*11*). Gas chromatographic determination of ethyl *N*-methylcarbamate and of NMF isotopomers was performed using a HP-5890A gas chromatograph with a nitrogen-selective detector, attached to a fused silica capillary HP-20M column (25 m × 0.32 mm i.d.; 0.32-μm film thickness). The column oven temperature was 130

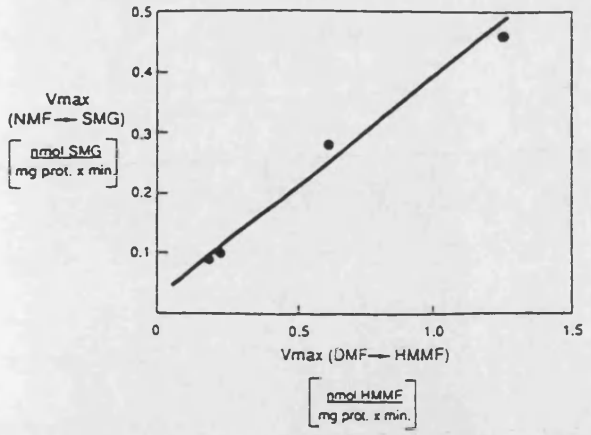

Figure 6. Correlation of apparent V_{max} of metabolism of NMF with that of DMF in microsomes from 4 human livers. The V_{max} values used are the ones which describe the enzyme with high affinity for NMF and DMF. For details of incubation and analysis of metabolites see the Experimental Procedures. Standard linear regression analysis gave a correlation coefficient of $r = 0.99$.

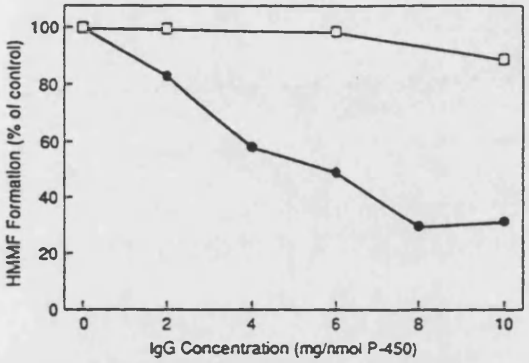

Figure 7. Effect of anti-rat P450 2E1 IgG (closed circles) or preimmune IgG (open squares) on the metabolism of DMF (10 mM) in human liver microsomes. Values, which are expressed as percentage of the rate of metabolic production of HMMF from DMF in incubations without IgG, are the mean of two individual microsomal preparations, each conducted in duplicate. Details of incubation conditions and metabolite analysis are described under Experimental Procedures.

metabolism of DMF was studied in incubates with microsomes in the presence of DEDTC which is a relatively specific inhibitor of P450 2E1 at concentrations in the 10^{-5}–10^{-4} M range (*34*). DEDTC indeed inhibited DMF oxidation potently (Figure 5B). Third, the rate of metabolism of DMF was compared with that of NMF, a P450 2E1 substrate (*20*), in liver microsomes from four different humans. The plot obtained (Figure 6) shows a good correlation between rates. Fourth, a monospecific antibody against rat P450 2E1 inhibited DMF metabolism in incubations with human liver microsomes substantially (Figure 7). Microsomal N-demethylation of aminopyrine was not affected by the presence of this antibody (result not shown). Fifth, in a preliminary experiment, HMMF was also detected in incubates of DMF (10 mM) with purified rat liver P450 2E1 after reconstitution with cytochrome P450 reductase and cytochrome b_5. The mixture was incubated for 1 h in order to maximize metabolite formation. The amount of HMMF generated, 110 nmol-(nmol of cytochrome P450)$^{-1}$, was 5 times that which was found under identical incubation conditions in microsomes from livers of streptozotocin-treated rats, which served as the source of the purified enzyme. The

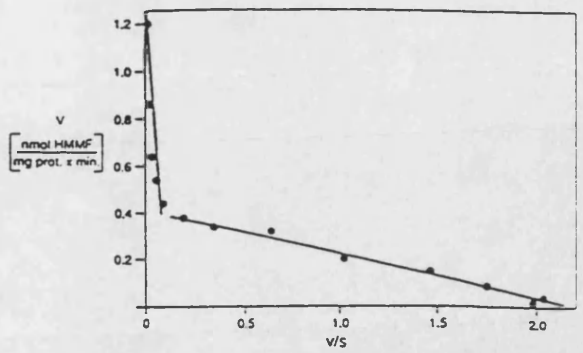

Figure 8. Eadie–Hofstee plot of generation of HMMF from DMF in incubates of rat liver microsomes. The individual values are the mean of 2 incubations. They were obtained with substrate concentrations ranging from 0.02 to 100 mM. The plot shown is representative of 4 experiments. Symbols "v" and "s" represent the reaction rate and substrate concentration, respectively. For details of incubation and metabolite analysis see the Experimental Procedures, and for the mean apparent K_m and V_{max} values see Table I.

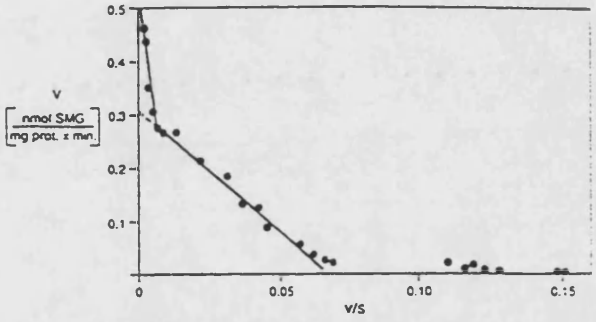

Figure 9. Eadie–Hofstee plot of metabolic generation of SMG from NMF in incubations of rat liver microsomes. The individual values are the mean of 2 incubations. They were obtained with substrate concentrations ranging from 0.2 to 200 mM. The plot shown is representative of 9 experiments. Symbols "v" and "s" represent the reaction rate and substrate concentration, respectively. For details of incubation and metabolite analysis see the Experimental Procedures. For the mean apparent K_m and V_{max} values see Table I.

difference in HMMF production between purified enzyme and microsomes mirrors the contribution of P450 2E1, as determined by *p*-nitrophenol oxidation, to the overall microsomal cytochrome P450 (result not shown).

Kinetic Analysis of Formamide Metabolism. In this series of experiments we wished to elucidate the discrepancy in the metabolic generation of *N*-methylcarbamic acid thioester from DMF between humans or animals in vivo and microsomal incubations in vitro, that is, to ascertain why the microsomal metabolism of DMF yields only HMMF but not SMG (*18*). To that end, DMF, NMF, and HMMF were incubated with rat and human liver microsomes. The rates of the enzyme-catalyzed reactions (i) DMF → HMMF, (ii) NMF → SMG, and (iii) HMMF → SMG were determined and used to construct Eadie–Hofstee plots. The plots for reactions i and ii are shown in Figures 8 and 9. They consist of two linear segments, which relate to low and high substrate concentrations and describe catalytic reactions of, respectively, high and low affinity for the enzyme. The apparent K_m and V_{max} values calculated from the high-affinity sections of the plots are shown in Table I. In the case of the metabolic formation of HMMF from DMF in rat liver microsomes (Figure 8) the high-affinity segment of the Eadie–Hofstee plot is

Table III. Intermolecular Kinetic Isotope Effect on DMF *N*-Methyl Oxidation

substrate concn (mM)			substrate concn (mM)		
DMF	[$^2H_6$]DMF	KDIE[a]	DMF	[$^2H_6$]DMF	KDIE[a]
2.5	2.5	5.8 ± 0.6	1	4	5.2 ± 0.1
0.5	0.5	4.6 ± 0.2	0.2	0.8	4.3 ± 0.2

[a] Ratio of formation of HMMF to [$^2H_5$]HMMF. Details of metabolite analysis are described under Experimental Procedures. Each value is the mean ± SD of 4 separate experiments. Mean KDIE ± SD of the mean values shown is 5.0 ± 0.7.

Table IV. Intramolecular Kinetic Isotope Effect on DMF *N*-Methyl Oxidation

[$^2H_3$]DMF concn (mM)	KDIE[a]	[$^2H_3$]DMF concn (mM)	KDIE[a]
5	5.7 ± 0.4	0.2	4.9 ± 0.2
1	5.0 ± 0.5		

[a] Ratio of formation of [$^2H_3$]HMMF to [$^2H_2$]HMMF. Details of metabolite analysis are described under Experimental Procedures. Each value is the mean ± SD of 4 separate experiments. Mean KDIE ± SD of the mean values shown is 5.2 ± 0.4.

[$^2H_1$]DMF. The ratios of V_{max}/K_m for DMF to V_{max}/K_m for either [$^2H_6$]DMF or [$^2H_1$]DMF gave 2.36 ± 0.74 (mean ± SD, n = 4) and 0.82 ± 0.33 (n = 5), respectively. The difference between these values is significant ($P < 0.01$). In order to measure the competitive intermolecular KDIE, mixtures of DMF and [$^2H_6$]DMF were subjected to incubation with rat liver microsomes. Each isotopomer was used at four different concentrations, and they were mixed at two concentration ratios (1:1 and 1:4). The quotient of the rates of production of HMMF, the metabolite of DMF, and of [$^2H_5$]HMMF, the metabolite of [$^2H_6$]DMF, at the different substrate concentrations ranged from 4.3 to 5.8, with a mean ± SD of 5.0 ± 0.7 (Table III).

For the determination of the intramolecular KDIE on the generation of HMMF from DMF, [$^2H_3$]DMF was synthesized and incubated with liver microsomes at three substrate concentrations. [$^2H_3$]HMMF and [$^2H_2$]HMMF were analyzed by GLC in the incubate as products of the oxidation of the N-CH_3 and the N-CD_3 groups, respectively (see Figure 2B). The KDIE calculated from the ratios of the isotopomeric metabolites gave 5.2 ± 0.4 as the mean ± SD (Table IV). It has to be stressed that we were able to conduct this study without the use of a mass spectrometer only because of the interesting finding that the GLC method allowed the separation of NMF and [$^2H_3$]-NMF (Figure 4), the products of hydrolysis either of HMMF and [$^2H_5$]HMMF, respectively, in the intermolecular competitive KDIE determination, or of [$^2H_2$]-HMMF and [$^2H_3$]HMMF, respectively, in the intramolecular KDIE measurement.

Effect of DMF, [$^2H_6$]DMF, and Related Amides on Metabolic Formyl Oxidation. The retarded excretion of AMCC as urinary metabolite of DMF (*11*, *16*) and the lack of generation of measurable SMG from DMF in vitro (*18*) suggest that metabolic formyl oxidation is inhibited by DMF or one of its products. This hypothesis was tested by the determination of metabolic production of SMG from NMF or HMMF in incubations with microsomes fortified with GSH and in the presence of varying concentrations of DMF. It is pertinent to reiterate that these experiments were possible only because DMF incubated on its own with microsomes did *not* yield detectable amounts of SMG (*18*). DMF did indeed inhibit the generation of SMG from NMF and HMMF. Figure 11 demonstrates that the inhibition of the oxidation of NMF by DMF was almost fully competitive. In all three repeat experiments the plot of the inhibited reaction provided apparent V_{max} values which were consistently slightly below the apparent V_{max} of the uninhibited reaction. Therefore, the possibility cannot be excluded that a minor noncompetitive component contributes to the overall mechanism of inhibition. The apparent K_i values calculated for DMF are 0.11 mM in rat liver microsomes (Table V) and 0.16 ± 0.06 mM in human liver microsomes (mean ± SD of the following individual values: 0.24, 0.09, 0.17, 0.15 mM). The apparent K_i values describing the ability of DMF to inhibit the metabolic formyl oxidation of HMMF are 0.48 ± 0.18 mM (mean ± SD, n = 3) in rat liver microsomes and 0.064 mM in microsomes from a human liver. These inhibition constants are thus similar to the apparent K_m values determined for the metabolic *N*-methyl oxidation of DMF, 0.20 and 0.12 mM, in rat and human microsomes, respectively (Table I).

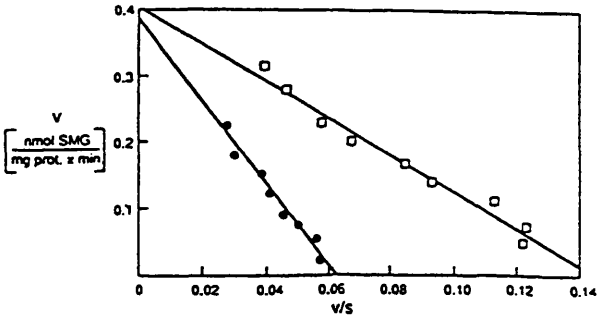

Figure 11. Eadie–Hofstee plot of metabolic generation of SMG from NMF in the absence (closed circles) and presence of DMF (open squares) in incubations of rat liver microsomes. The individual values are the mean of 2 incubations. They were obtained with substrate (NMF) concentration ranging from 0.4 to 10 mM and inhibitor (DMF) concentrations of 0.1 mM. The plot shown is representative of 3 experiments. Symbols "v" and "s" represent the reaction rate and substrate concentration, respectively. For details of incubation and metabolite analysis see the Experimental Procedures. The apparent K_i value for the inhibition of NMF oxidation by DMF computed from this experiment is 0.08 mM.

Table V. Inhibition of Metabolic Generation of SMG from NMF in Rat Liver Microsomes by Low-Molecular-Weight Amides

inhibitor $R_1R_2NC(O)R_3$				
R_1	R_2	R_3	app K_i (mM)[a]	n[b]
H	H	H	44 ± 7	4
C_2H_5	H	H	0.34 ± 0.09	3
CH_3	CH_3	H	0.11 ± 0.02	9
C_2H_5	C_2H_5	H	0.054 ± 0.027	4
C_4H_9	C_4H_9	H	0.56 ± 0.14	4
H	H	CH_3	158 ± 11	3
CH_3	H	CH_3	6.52 ± 1.16	3
CH_3	CH_3	CH_3	0.25 ± 0.04	3
C_2H_5	C_2H_5	CH_3	2.51 ± 0.33	3

[a] Details of metabolite analysis and calculation of apparent K_i values are described under Experimental Procedures. Values are the mean ± SD. [b] Number of separate experiments, each of which consisted of 6–8 individual incubations.

Next we wanted to test the hypothesis that the ability of DMF to inhibit metabolic formyl oxidation and thus the generation of SMG from NMF is subject to a KDIE. To that end, [$^2H_6$]DMF at several concentrations was added to incubates of NMF with rat liver microsomes. The K_i value obtained was 0.16 ± 0.02 mM (mean ± SD

to DMF in the work environment (*38–41*). It is unlikely that DMF itself acts like disulfiram, as it does not inhibit alcohol or aldehyde dehydrogenase enzymes in vitro (*42, 43*). Instead, a DMF metabolite might inhibit acetaldehyde dehydrogenases. In this context it is instructive to consider the structural similarity between disulfiram [$(C_2H_5)_2NC(S)SSC(S)N(C_2H_5)_2$] or its analog dioxiram [$(C_2H_5)_2NC(O)SSC(O)N(C_2H_5)_2$] and the DMF metabolites SMG and AMCC. Both disulfiram and dioxiram at concentrations as low as 2 μM were shown to decrease potently the activity of sheep liver cytoplasmic aldehyde dehydrogenase in vitro (*44*). In view of their structural resemblance with disulfiram and dioxiram SMG and AMCC might likewise inhibit aldehyde detoxification and thus be responsible for the alcohol incompatibility caused by DMF. Alternatively, the interaction responsible for the DMF–alcohol incompatibility reaction might occur directly at the level of hepatic P450 2E1. P450 2E1 was recently shown to catalyze the oxidation of acetaldehyde with a K_m of about 30 μM (*45*). Thus DMF itself might increase hepatic levels of acetaldehyde via inhibition of P450 2E1, even though the contribution of this enzyme to the overall metabolism of acetaldehyde in vivo is probably small. These speculations and their relevance to the mechanism of the adverse effects of DMF need further experimental verification.

The ability of DMF to inhibit the P450 2E1-mediated metabolism of its own metabolites is consistent with the observation that DMF blocks the N-demethylation of *N,N*-dimethylnitrosamine, another P450 2E1 substrate (*21*). The K_i value reported in that paper, 0.07 mM, is close to that reported here for the inhibition by DMF of NMF formyl oxidation (0.11 mM). It is remarkable that the inhibitory potential of DMF on the metabolism of substrates of P450 2E1 was first discovered thirty years ago. Heath showed then that DMF retards the exhalation in rats of $^{14}CO_2$ derived from [^{14}C]-labeled *N,N*-dimethylnitrosamine (*46*). His astute deduction that both DMF and *N,N*-dimethylnitrosamine interact with the same oxidizing enzymes because they share structural similarity has been prophetic in view of the conclusion proffered here that both molecules are substrates of P450 2E1. The microsomal hydroxylation of DMF, like that of *N,N*-dimethylnitrosamine, is characterized by both a high- and a low-affinity component. The low-affinity segment of the oxidation of *N,N*-dimethylnitrosamine has been attributed to P450 LM2 (*19*). The findings described above of the influence of substitution of hydrogen in the DMF molecule by deuterium are somewhat at variance with the effect of deuterium substitution on the metabolic N-demethylation of *N,N*-dimethylnitrosamine. Deuteration of the methyl moieties in *N,N*-dimethylnitrosamine increased the apparent K_m by a factor of 5, but did not change the apparent V_{max} of the reaction (*47*), whereas in our experiments deuteration of the DMF methyl moieties affected the V_{max} but not the K_m.

The intriguing inhibition by DMF of metabolic formyl oxidation explains why the start of the urinary excretion of AMCC after exposure to DMF in vivo is delayed (*11, 16*). This explanation hints at the possibility that manifestations of detrimental effects of DMF on the health of workers after overexposure may develop well beyond the end of the work shift. Inhibition of the metabolism of NMF by DMF also rationalizes the observation that DMF decreased the severity, and postponed the onset, of NMF-induced hepatotoxicity as measured by elevated serum sorbitol dehydrogenase levels in rats (*17*).

Another vexing question associated with the link between DMF toxicity and its metabolic conversion to *N*-methylcarbamic acid thioesters has been whether HMMF is a substrate for the formyl-oxidizing microsomal enzyme, or if production of SMG, the progenitor of the ultimate metabolite AMCC, from DMF (*10, 11*) requires the intermediacy of NMF. NMF is only a quantitatively minute urinary metabolite of DMF, whereas its relatively stable *N*-(hydroxymethyl) precursor HMMF is the major biotransformation product of DMF in the urine (*7–9*). In view of this fact, it has been impossible to decide whether NMF and not HMMF is the immediate metabolic precursor of SMG and AMCC in vivo. The interpretation of previous studies on the metabolism of HMMF in microsomes in vitro (*18*) has been severely confounded by the fact that the product of chemical synthesis of HMMF from NMF and formaldehyde always contained at least 5% NMF, so that it has never been clear whether SMG formation reflected the metabolism of HMMF or that of its contaminant NMF. In contrast, in the investigations described above we have been able to use authentic HMMF which contained only up to 0.5% NMF. The generation of SMG proceeded at a rate much inferior to that observed with NMF as substrate; for example, in the case of rat liver microsomes the rate was only 5% of that discerned with NMF. The knowledge of the kinetic parameters which characterize these metabolic processes (Table I) allows calculation according to which the rate of SMG production from HMMF was approximately 3 times as fast as that expected if the 0.5% contaminant NMF was the exclusive substrate of microsomal metabolism. The possibility cannot be completely excluded that microsomes catalyze the breakdown of HMMF to NMF. The HPLC method used here for the simultaneous measurement for NMF and HMMF did not clarify this matter conclusively as components of the microsomal incubate interfered with the determination of NMF. However, the finding that the plot of v against s for the biotransformation HMMF → SMG observed saturation kinetics (result not shown) renders the possibility unlikely that NMF generated by degradation of HMMF, rather than HMMF itself, was responsible for the production of SMG. The kinetic parameters help also to explain why the formation of SMG has never been observed in incubations of liver microsomes with DMF. Under the conditions of incubation adopted in the study reported here 10 mM DMF would generate not more than 20 μM HMMF within 30 min. Assuming an (obviously fictitious) constant presence of 10 μM HMMF during the whole of the incubation period the final concentration of SMG generated from DMF via HMMF would be 3 nM. As the limit of detection of the assay used is about 0.1 μM, it is not surprising that SMG was not found in these incubations, even without taking the ability of DMF to inhibit SMG formation into consideration.

In enzymic systems, isotope effects can be measured using several different experimental designs, which furnish intermolecular or intramolecular KDIE values. For the measurement of the intermolecular KDIE one can adopt a noncompetitive and a competitive approach. The noncompetitive design requires separate determinations of V_{max} and K_m for both labeled and unlabeled substrate. In the competitive strategy, a known mixture of labeled

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PUBLICATION 56

Unusual Deuterium Isotope Effect on the Retention of Formamides in Gas Liquid Chromatography

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Short Communication

Unusual deuterium isotope effect on the retention of formamides in gas–liquid chromatography

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ABSTRACT

The deuterium isotope effect on gas chromatographic retention of a series of N,N-dimethylformamide, N-methylformamide and formamide isotopomers was studied on methyl and phenyl methyl polysiloxane and polyethylene glycol stationary phases. The deuterium isotope effect was substantially affected by the position of deuterium in the molecule. Whereas methyl-deuterated $(C^2H_3)_2NCHO$ and C^2H_3NHCHO eluted, as expected, earlier than their non-labelled analogues, elution of formyl-deuterated $(CH_3)_2NC^2HO$, CH_3NHC^2HO and NH_2C^2HO was always delayed. The presence of chemically inequivalent deuterium atoms in $(C^2H_3)_2NC^2HO$ and $C^2H_3NHC^2HO$ resulted in final deuterium isotope effects to which the contributions of the individual groups were additive. The elution order of the individual isotopomers was not affected by the polarity of the stationary phase. Interpretation of the deuterium isotope effect observed is provided by the theory of vapour pressure isotope effects.

INTRODUCTION

Changes in the chromatographic retention of molecules with different isotopic composition have often been noted. Perhaps most reports deal with the effect of replacing ^1H by ^2H (deuterium) (for review, see ref. 1). The great majority of reported gas chromatographic (GC) separations result in earlier elution of heavier ^2H-labelled compounds, which is referred to as the inverse isotope effect [1]. Accordingly, in a series of isotopomers differing by a number of deuterium atoms within some structural unit, *e.g.* in an alkyl group [2,3] or on an aromatic ring [4], the compounds are eluted in the order of decreasing number of deuterium atoms. The normal isotope effect, *i.e.* later elution of heavier species, is observed mainly in absorption GC (GSC), for example on alkali-etched glass capillary columns [5,6]. Examples of normal isotope

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CH_3CH_3NHCHO	d_0-DMF	CH_3NHCHO	d_0-MF
$CH_3CH_3NHC^2HO$	d_1-DMF	CH_3NHC^2HO	d_1-MF
$CH_3C^2H_3NHCHO$	d_3-DMF	C^2H_3NHCHO	d_3-MF
$C^2H_3C^2H_3NHCHO$	d_6-DMF	$C^2H_3NHC^2HO$	d_4-MF
$C^2H_3C^2H_3NHC^2HO$	d_7-DMF		
		NH_2CHO	d_0-F
		NH_2C^2HO	d_1-F

Fig. 1. Structures of DMF, MF and F isotopomers.

effects in partition GC (GLC) are very scarce [3,5].

In this paper we report on an unusual observation, namely that the deuterium isotope effect on GC retention may be substantially affected by the position of deuterium atoms in the molecule. The observation is discussed in terms of the theory of vapour pressure isotope effects.

EXPERIMENTAL

Chemicals

Unlabelled N,N-dimethylformamide (d_0-DMF), N-methylformamide (d_0-MF) and formamide (d_0-F) were bought from Aldrich (Gillingham, UK), d_7-DMF was bought from Sigma (Poole, UK). Compounds d_6-DMF, d_3-DMF and d_3-MF were prepared from d_6-dimethylamine hydrochloride (Sigma), d_3-dimethylamine hydrochloride (a gift from Dr. R.P. Hanzlik, University of Kansas) and d_3-methylamine hydrochloride (Sigma), respectively, and ethyl formate [7]. By analogy, d_1-DMF, d_1-MF and d_1-F were prepared from the respective amine hydrochlorides and d_1-methyl formate (Aldrich). Compound d_4-MF was prepared from d_3-methylamine hydrochloride and d_1-methyl formate. N,N-Dimethylacetamide (DMA) was bought from Aldrich; propyl-N-methylcarbamate was prepared as described previously [8]. For structures of DMF, MF and F isotopomers see Fig. 1.

GC analysis

Retention times were measured on an HP-5890 A gas chromatograph with 3394 A integrator. The fused-silica capillary columns used were HP-20M (polyethylene glycol 20M), 25 m × 0.32 mm I.D., 0.3 μm film thickness, and HP-50 + (cross-linked 50% phenyl methyl silicone gum), 15 m × 0.53 mm I.D., 1.0 μm film thickness, both from Hewlett-Packard, and DB-1 (cross-linked 100% methyl silicone gum), 15 m × 0.53 mm I.D., 3.0 μm film thickness, from J & W Scientific. The injector was in a split mode with a 1:30 splitting ratio, and the nitrogen-selective detector was used. Helium was employed as carrier gas. Experimental conditions of the measurements and informative values of dead and retention times are shown in Table I.

A typical sample was a solution of a single isotopomer (1 m*M*) and an internal standard (1 m*M*) in acetone (1 μl). The internal standards were DMA for DMF, propyl-N-methylcarbamate (on HP-20M) or DMA (on HP-50 + and DB-1) for MF, and MF for F.

TABLE I

EXPERIMENTAL CONDITIONS OF GAS CHROMATOGRAPHIC MEASUREMENTS

	HP-20M				HP-50+				DB-1			
	Temp. (°C)	Flow-rate (ml/min)	t_M (min)	t_r[a] (min)	Temp. (°C)	Flow-rate (ml/min)	t_M (min)	t_r[a] (min)	Temp. (°C)	Flow-rate (ml/min)	t_M (min)	t_r[a] (min)
DMF	90	0.9	1.9	7.3	75	2.1	1.4	5.1	75	2.3	1.2	4.8
MF	130	0.7	2.0	7.7	75	2.1	1.4	5.0	75	2.3	1.2	4.3
F	130	0.7	2.0	12.1	75	2.1	1.4	3.5		Not measured[b]		

[a] t_R of the non-deuterated isotopomer.

[b] Not measured because of the strong tailing and dose dependence of the retention time.

Calculations

In this study, the deuterium isotope effect on chromatographic retention is expressed using relative retention, r_{12}, between a deuterated compound and its non-deuterated analogue: $r_{12} = t'_R(\text{deuterated})/t'_R(\text{non-deuterated})$. Here $t'_R = t_R - t_M$, where t_R is retention time, t'_R is corrected retention time and t_M is dead time. In the most straightforward way, r_{12} can be measured upon injection of both compounds together. This could not be applied here since in some cases peaks of isotopomers could not be resolved sufficiently. Thus, separate injection of each compound was necessary. The absolute values of retention time, however, may be affected by the drift or fluctuations in experimental conditions during measurement. On the other hand, accuracy of the retention parameters determined should be very high. Therefore, a retention of each isotopomer (i) including the non-deuterated compound was related to the retention of an internal standard (I.S.), which was added for this purpose to the sample. Auxiliary relative retentions r_{12aux} were calculated as $r_{12aux} = t'_R(\text{i})/t'_R(\text{I.S.})$ and, finally, the r_{12} value for each deuteromer was obtained:

$$r_{12} = r_{12aux}(\text{deuterated})/r_{12aux}(\text{non-deuterated})\ .$$

RESULTS AND DISCUSSION

The deuterium isotope effect on the GC retention of a series of deuteromers of DMF, MF and F was studied on three fused-silica capillary columns of different polarity. As an index of the deuterium isotope effect, values of the relative retention r_{12} of a pair of deuterated/non-deuterated isotopomers were measured. The results (Table II) are consistent for DMF, MF and F, and can be summarized as follows.

(a) The presence of deuterium in the methyl position of formamides results in lower retention.

(b) The presence of deuterium in the formyl position of formamides results in higher retention. The formyl-related deuterium isotope effect is more pronounced than the methyl-related deuterium isotope effect: the corresponding values of an index $(r_{12} - 1)/N_D$ (where N_D is number of deuterium atoms in the molecule) were 0.009 to 0.024 and −0.001 to −0.008, respectively. The separation of MF isotopomers is shown in Fig. 2.

TABLE II

DEUTERIUM ISOTOPE EFFECT ON THE RETENTION OF N,N-DIMETHYLFORMAMIDE, N-METHYLFORMAMIDE, AND FORMAMIDE ISOTOPOMERS

Compound	r_{12} [a]		
	HP-20M	HP-50+	DB-1
d_0-DMF	1.000	1.000	1.000
d_1-DMF	1.023	1.013	1.009
d_3-DMF	0.998	0.989	0.990
d_6-DMF	0.995	0.979	0.982
d_7-DMF	1.017	0.991	0.989
d_0-MF	1.000	1.000	1.000
d_1-MF	1.017	1.014	1.010
d_3-MF	0.982	0.977	0.980
d_4-MF	1.000	–	–
d_0-F	1.000	1.000	–[b]
d_1-F	1.019	1.024	–[b]

[a] The values are mean of at least eight measurements; S.D. was less than 0.001 in most cases.

[b] Not measured because of the strong tailing and dose dependence of retention time.

(c) The presence of chemically inequivalent deuterium atoms results in the final deuterium isotope effect, to which the contributions of individual deuterium atoms are additive. Thus, r_{12} of d_7-DMF or d_4-MF is consistent with that

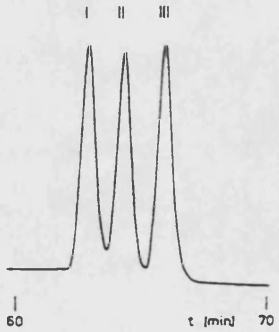

Fig. 2. Resolution of C^2H_3NHCHO (I), CH_3NHCHO (II) and CH_3NHC^2HO (III) on fused-silica capillary column HP-20M 25 m × 0.32 mm I.D., 0.3 μm film thickness; column temperature 70°C; carrier gas helium, 1.0 ml/min.

estimated from the individual r_{12} values of partially deuterated analogues d_6-DMF and d_1-DMF, or d_3-MF and d_1-MF, respectively.

(d) The observations described under (a) to (c) are similar on strongly polar (HP-20M), slightly polar (HP-50+), and low polar (DB-1) columns.

Numerous reports on GC of isotopomers are consistent in that the deuterium isotope effect related to deuterium in methyl or methylene groups is regularly the inverse effect [1,9,10]. This has been interpreted to be a consequence of shorter C–^{2}H internuclear distances as compared with C–H bonds, resulting in lower molar volumes and thus weaker dispersion forces. The effect on dispersion forces is thus responsible for the inverse deuterium isotope effect related to C^2H_3 groups in DMF and MF (even when the contribution of these forces to overall interaction does not have to be prevailing). Dispersion forces seem to predominate in the interaction of formamides with non-polar stationary phases such as DB-1, as judged from the elution in the order of F, MF and DMF, *i.e.* with increasing molar volume. In contrast, these compounds are eluted in the opposite order where polar interactions (dipole–dipole, dipole–induced dipole, etc.) prevail, as in the case of elution from polar HP-20M column. The increasing role of polar interactions in the order of DMF to F is consistent with the order of boiling points: DMF, 153°C; MF, 185°C; F, 195°C.

Unlike methyl-deuterated compounds, the normal DIE as observed with formyl-deuterated formamides seems to be surprising and difficult to account for by simple considerations. The key to understanding this phenomenon is provided by the theory of isotope effects, especially vapour pressure isotope effects, supported by numerous experimental data (for review, see ref. 11). All available reports show that deuterium at saturated carbon displays inverse vapour pressure isotope effect, whereas substitution in groups that form hydrogen bonds and associate often results in normal vapour pressure isotope effect. There is an intrinsic relationship between isotope effect and molecular motion; normal isotope effects correlate with blue and inverse isotope effects with red frequency shifts.

Good examples to study the vapour pressure isotope effect related to deuterium in different groups of the same compound are methylacetylenes and alkylamines [12–14]. Here, deuterium in the methyl group results in an inverse vapour pressure isotope effect, whereas CH_3CC^2H, $CH_3N^2H_2$ and $(CH_3)_2N^2H$ exhibit a normal vapour pressure isotope effect. The above effects were interpreted with the aid of Bigeleisen theory [15]. In a most simplified way, the vapour pressure isotope effect is calculated using the equation $\ln P'/P = A/T^2 - B/T$, where P' and P are the vapour pressures of the heavier and lighter isotopomers, respectively, A (lattice term) and B (zero-point energy term) represent external and internal frequency modes, respectively, and T is temperature. In this model, replacement of H by ^{2}H in the methyl group affects only the A term, which is always positive so that inverse vapour pressure isotope effects occur. On the other hand, the location of deuterium in methynic or amino groups affects both A and B terms, the latter being responsible for the normal vapour pressure isotope effect observed. Analysis of the shape of an experimental plot of $\ln P'/P$ *vs.* T for CH_3CC^2H, $CH_3N^2H_2$ and $(CH_3)_2N^2H$ revealed temperature-dependency of B, which is indicative of molecular association in condensed phases [12–14]. This view was further reinforced by finding that the vapour pressure isotope effect as measured for $CH_3N^2H_2$ or $(CH_3)_2N^2H$ in solution with hexane changed from a normal to inverse effect at high dilution [13,14], in which the association was negligible.

The data on formamides presented here are obviously in good agreement with those on methylacetylenes and amines. Thus, the normal isotope effect related to GC retention of formyl-deuterated formamides is likely to reflect stronger interaction of those compounds, via the formyl group (compared with their non-labelled counterparts), with each other as well as with other molecules such as GC stationary phases. Owing to the polar nature of those interactions one can expect that the magnitude of the deuterium isotope effect on GC retention of formyl-deuterated formamides will increase with polarity of the stationary phase. This was in fact the case, except for F (Table II). Another similarity was observed between the sets of

methylacetylene, methyl- and dimethylamine and formamide isotopomers, namely that the presence of chemically inequivalent deuterium atoms resulted in a final deuterium isotope effect to which contributions of individual deuterium atoms were additive.

In conclusion, the deuterium isotope effect on GC retention of formamides reported here, despite its unusual character, complies well with the general theory of isotope effects.

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PUBLICATION 57

Synthesis of the Enantiomers of the Bioreductively-Activated Cytotoxin RSU 1069 and its Pro-Drug RB 6145 and Lack of Stereoselectivity in their Cytotoxicity and Radiosensitization *in Vitro*

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SYNTHESIS OF THE ENANTIOMERS OF THE BIOREDUCTIVELY-ACTIVATED CYTOTOXIN RSU-1069 AND ITS PRODRUG RB6145 AND LACK OF STEREOSELECTIVITY IN THEIR CYTOTOXICITY AND RADIOSENSITIZATION *IN VITRO*

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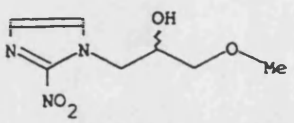
The R(+)- and S(−)-enantiomers of the radiosensitizer and bioreductively-activated cytotoxin RSU-1069 and their prodrugs have been synthesised. The parent drugs were evaluated as radiosensitizers and bioreductively-activated cytotoxins *in vitro*. No stereoselectivity in the activities *in vitro* of the two enantiomers was evident and both compounds were potent hypoxia-selective agents.

KEY WORDS: Bioreductive, cytotoxin, radiosensitizer, RSU-1069, RB6145, enantiomers

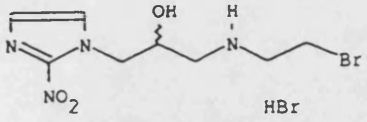
INTRODUCTION

The dual-function radiosensitizer and bioreductively-activated cytotoxin α-[[(2-bromoethyl)amino]methyl]-2-nitro-1*H*-imidazole-1-ethanol hydrobromide (1, RB6145) is the lead compound that has arisen from a programme of development of 2-nitroimidazole derivatives as agents for cancer chemotherapy.[1,2] Selective toxicity towards hypoxic cells within tumours is due to anaerobic, enzymatic reduction of the drugs to toxic metabolites. In contrast, the radiosensitizing action of these compounds is through radiation-induced fast free-radical mechanisms occurring within normally radiation resistant hypoxic cells.

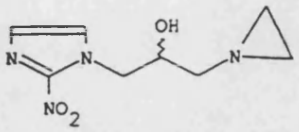
RB6145 (1) is a prodrug of RSU-1069 (2)[3,4]—the most effective of a series of dual-function compounds related to misonidazole, a drug withdrawn from clinical evaluation owing to cumulative neurotoxicity.[5] A number of regioisomers of 2 have been evaluated[6] but the prodrug 1 remains the lead compound and has now progressed to the status of a candidate for clinical evaluation. However, to date stereoisomerism



Misonidazole



RB6145 (1)



RSU-1069 (2)

has not been addressed, and all biological evaluation of the drug both *in vitro* and *in vivo* has been carried out using the racemic mixture of the R(−) and S(+)-enantiomeric forms.

A possible clinical future for **1** means that it has become necessary to evaluate thoroughly the separate enantiomers for their individual cytotoxic properties. In view of the enzymatic nature of the bioreductive activation, differences in their rates of reduction by the cellular reductases involved could not be discounted. Similarly differences in reactivity of the enantiomeric forms of various metabolites towards cellular targets such as DNA are possible.

We therefore report here on the synthesis of the respective enantiomers of **2** and their prodrugs, and a preliminary study comparing the efficacies of the parent drugs as bioreductively-activated cytotoxins and as radiosensitizers *in vitro*.

CHEMISTRY

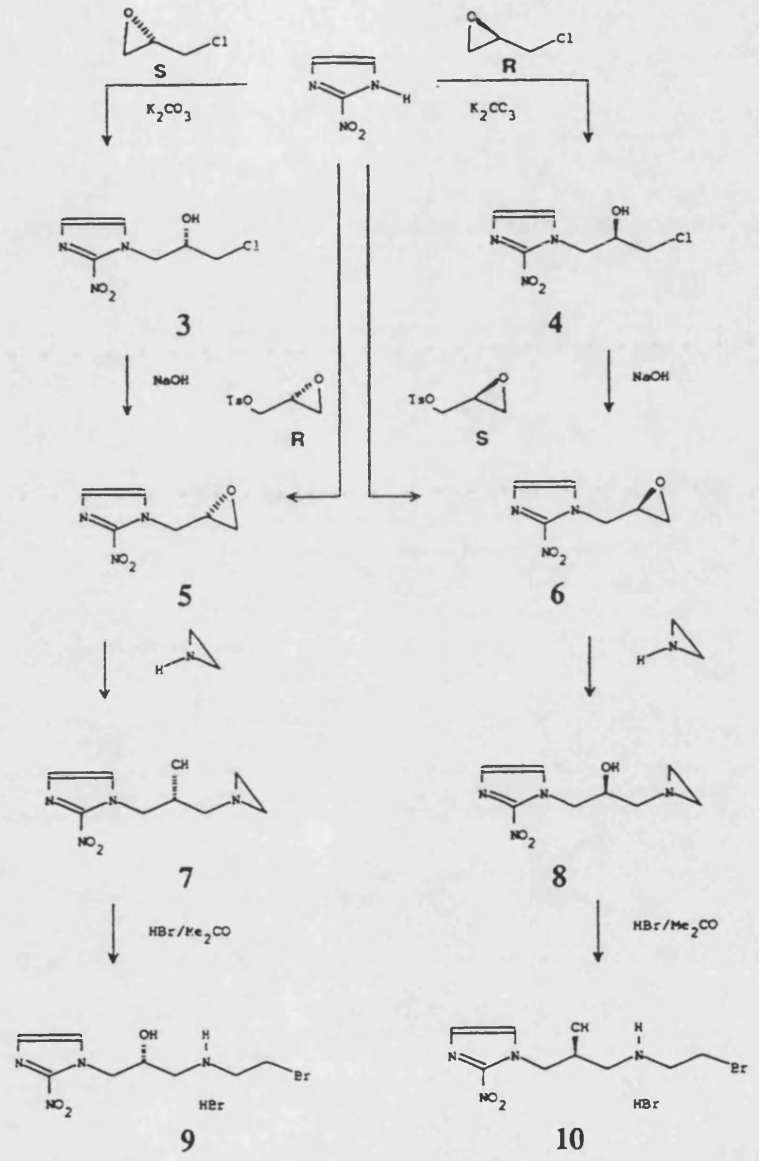
Two routes to the enantiomers **7** and **8** were evaluated. The first (A) makes use of the chiral forms of epichlorohydrin. Reaction with 2-nitroimidazole (azomycin) affords the chiral chlorohydrins **3** and **4** which, upon treatment with aqueous base give the oxiranyl synthons **5** and **6**. These compounds were then converted into the corresponding aziridine derivatives **7** and **8** essentially as described by us earlier.[2]

The second route (B) utilises the respective enantiomers of oxiranyl methyl tosylate, which react preferentially with the anion of 2-nitroimidazole by nucleophilic displacement of the tosylate rather than by epoxide ring-opening. This was confirmed from the resulting aziridines which were identical to those obtained by Route A. The aziridines **7** and **8** were converted into their respective ring-opened hydrobromides with HBr/Me_2CO to afford the hydrobromides **9** and **10**. These chiral prodrug forms of **2** were used in the determination of enantiomeric purity by chiral HPLC. Compounds **9** and **10** were found to be 99.3 and 97.1% enantiomerically pure respectively.

EXPERIMENTAL SECTION

Synthetic Chemistry

NMR spectra were obtained at 60 MHz with a Jeol PMX60SI spectrometer using $SiMe_4$ as internal standard. Elemental microanalyses were carried out by Butterworth



Scheme 1 Synthetic routes to enantiomers of 1.

Laboratories Ltd., Middlesex, U.K. HPLC experiments were carried out with a Diacel AS column (5 μ, 0.46 × 25 cm) precooled and maintained at 10°C with a mobile phase of EtOH/Hexane (1:9, v/v). Detection was at 315 nm. Optical rotations were determined on an Optical Activity Ltd AA10 automatic polarimeter.

(R)(−)-α-(1-aziridinylmethyl)-2-nitro-1-H-imidazole-1-ethanol (7)
Route A: A mixture of powdered 2-nitroimidazole (0.42 g, 3.7 mmol), 5 mL of (2S)(+) epichlorohydrin and anhydrous K_2CO_3 (0.085 g, 0.062 mmol) was stirred under reflux for 10 min., filtered while hot and any insoluble material washed with a little hot EtOH. From the refrigerated filtrate and concentrated remainder (2S)-α-(chloromethyl)-2-nitro-1*H*-imidazole-1-ethanol (3) (0.56 g, 66%) was obtained after recrystallization from EtOH as pale yellow crystals: mp 153–154°C. This material was stirred vigorously at 20°C for 0.5 h in aqueous NaOH (10%, 3 mL). Cold water (5 mL) was then added, and the solution was extracted with $CHCl_3$ (3 × 10 mL). The combined extracts were dried and evaporated and the residue was recrystallized from water and dried to give (2S)-2-nitro-1-(2-oxiranylmethyl)-1*H*-imidazole (5) (0.3 g, 73%) as a white solid: mp 54–55°C. The above material (0.3 g, 1.8 mmol) was dissolved in EtOH (3.5 mL, containing 1% Et_3N) and aziridine (0.24 g, 5.4 mmol) was added (**CAUTION**). The solution was heated under gentle reflux for 10 min. and then cooled and evaporated. The residue was recrystallized from EtOH (1% Et_3N) to afford (R)(−)-α-(1-aziridinylmethyl)-2-nitro-1*H*-imidazole-1-ethanol (7) (0.22 g, 57%) as white crystals: mp 119.5–121°C. NMR ($CDCl_3$) δ 1.3 (dd, 2H, J = 2 and 4 Hz, aziridine CH_2), 1.8 (dd, 2H, J = 2 and 4 Hz, aziridine CH_2), 2.4 dd, 2H, J = 4 and 7.5 Hz, CH_2-aziridine), 4.2 (m, 2H, C*H*O*H*), 4.3 (dd, 1H, J = 7.2 and 16.8 Hz) and 4.7 (dd, 1H, J = 2 and 9.6 Hz) Im-CH_2, 7.1 (s, 1H, Im-H), and 7.2 (s, 1H, Im-H) ppm. Anal. Calc. for $C_8H_{12}N_4O_3$: C, 45.28; H, 5.66; N, 26.42%. Found: C, 45.51; H, 5.76; N, 26.04%.

$$[\alpha]_D^{24} = -23.5°(c = 1.15, CHCl_3).$$

Route B: 1-Potassio-2-nitroimidazole was prepared by treatment of 2-nitroimidazole with one equivalent of potassium tert-butoxide in DMF, heating at 110°C for 0.5 h, cooling and washing the resulting solid with dry CH_2Cl_2. 1-Potassio 2-nitroimidazole (0.75 g, 5 mmol) was dissolved in anhydrous DMF (10 mL) together with (2R)(−) glycidyl tosylate (1.6 g, 7 mmol) and the solution stirred for 12 h at ambient temperature. The solvent was removed under reduced pressure at 35°C and the residue redissolved in 100 mL $CHCl_3$, washed with water, dried and evaporated. The residue was purified by flash chromatography on silica (200 g), eluting with CH_2Cl_2 to give (5) (0.58 g, 55%) identical to that obtained by Route A. This material was trreated with 1(H)-aziridine as described above to obtain material identical to 7 above.

(R)-α-[[(2-Bromoethyl)amino]methyl]-2-nitro-1H-imidazole-1-ethanol hydrobromide (9)
Compound 7 was treated with aqueous HBr in acetone as described previously,[2] to give the hydrobromide 9 in 74% yield: mp 149–150.5°C (Lit.[2](±) 150–151°C), 99.3% optically pure by HPLC (t_r = 27.4 min.).

(S)(+)-α-(1-Aziridinylmethyl)-2-nitro-1H-imidazole-1-ethanol (8)
This enantiomer was prepared exactly as described above from either (2R)(−) epichlorohydrin or (2S)(+) glycidyl tosylate to give 8: mp 118.5–120°C. The NMR spectra was identical to that obtained for 7 at 60 MHz. Anal. Calcd. for $C_8H_{12}N_4O_3$: C, 45.28; H, 5.66; N, 26.42%. Found: C, 45.53; H, 5.81; N, 26.26%.

$$[\alpha]_D^{24} = +21.4°(c = 0.98, CHCl_3).$$

(S)-α-[[(2-Bromoethyl)amino]methyl]-2-nitro-1H-imidazole-1-ethanol (**10**)
The prodrug (**10**) was prepared in the same manner as **9** and as described previously:[2] mp 148–149°C (Lit.[2](±) 150–151°C), 97.1% optically pure by HPLC (t_r = 23.05 min.).

Biological Studies

In vitro evaluation of the compounds as bioreductively-activated cytotoxins and as radiosensitizers was carried out as described previously using V79-379A cells.[6,7,8] Selective toxicity to these cells under hypoxic conditions was determined using the MTT assay.[7] These results are presented in Figure 1. Cytotoxicity towards cells is presented as a reduction in optical density, which is proportional to the number of viable cells in this assay, plotted against drug concentration. The concentration required to kill 50% of the aerobic cells (C_{50}(air)) under the conditions of the assay, divided by the concentration required to kill hypoxic cells ($C_{50}(N_2)$), was thus 20 for both enantiomers **7** and **8** and for the racemate **2**.

These compounds were also assayed for their ability to sensitize hypoxic V79 cells *in vitro* to γ-radiation (^{60}Co) carried out as described previously.[8] The surviving fraction of these cells is plotted as a funtion of dose (Gy) for various concentrations of the drugs and compared with the effects of radiation alone under hypoxic conditions. Enhancement ratios are then determined (ratio of survival curve slope, drug treated versus control). The combined results are then presented as shown in Figure 2. There

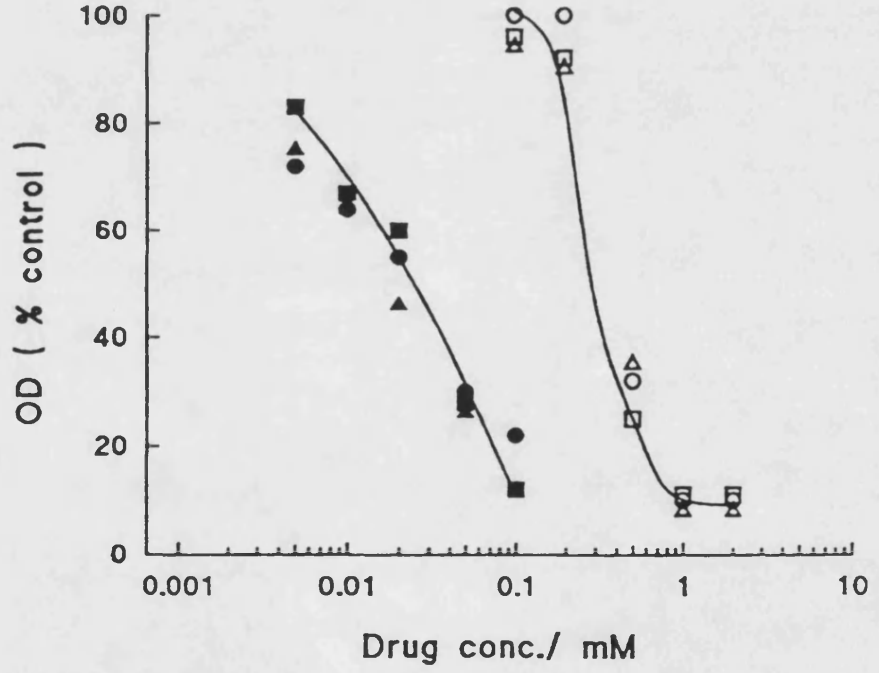


Figure 1 The *in vitro* cytotoxicity of compounds **2** (●), **7** (▲) and **8** (■) under hypoxic (closed symbols) and aerobic (open symbols) conditions.

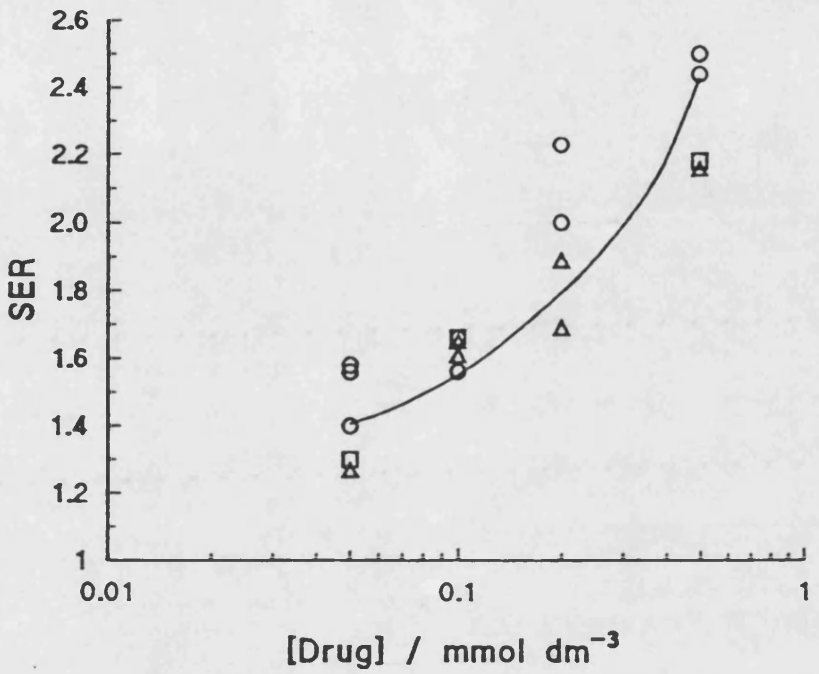


Figure 2 Dependence of hypoxic radiosensitization of V79 cells *in vitro* on the concentration of 2 (○), 7 (△) and 8 (□).

was no difference in radiosensitizing efficiency between the two enantiomers 7 and 8 and results for both compounds were comparable to those of the racemic drug.

CONCLUSIONS

Stereospecific synthesis of the R(+) and S(−) enantiomers of the 2-nitroimidazole aziridine RSU-1069 (2) has been achieved by two different routes from stereoisomeric precursors. No racemisation occurs during these synthetic routes and ring-opened prodrug forms of the two aziridinyl compounds were greater than 97% enantiomerically pure by chiral HPLC analysis. The two enantiomers do not show any differences in their hypoxia-selective cytotoxicity *in vitro*, nor do they exhibit any significant differences in their radiosensitizing efficiencies. A similar lack of stereoselectivity in the pharmacokinetics and metabolism *in vivo* cannot be assumed, although this is the case with the related developmental hypoxic cell radiosensitizer pimonidazole.[9] Full studies *in vivo* using the prodrugs **9** and **10** are to be reported elsewhere.

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PUBLICATION 58

The Reaction of 1,1,1,5,5,5-Hexafluoropentane-2,4-dione with Hydrazines: a Reinvestigation

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The reaction of 1,1,1,5,5,5-hexafluoropentane-2,4-dione with hydrazines: a re-investigation

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Abstract

The reaction of 1,1,1,5,5,5-hexafluoropentane-2,4-dione with hydrazine (N_2H_4) in boiling ethanol gives 3,5-bis(trifluoromethyl)pyrazole but reaction with *N*-aryl or *N*-aroyl hydrazines gives the 1-aryl- or 1-aroyl-3,5-bis(trifluoromethyl)-4,5-dihydro-5-hydroxypyrazoles, as shown by NMR and mass spectra, in contrast to a previous report.

Introduction

During a programme of synthesis of (trifluoromethyl)heterocycles for medicinal and pH sensor applications [1], 3,5-bis(trifluoromethyl)pyrazole (**4a**) and its 1-substituted analogues were required. Preparation of the parent compound **4a** has been reported to have been carried out by reaction of 1,1,1,5,5,5-hexafluoropentane-2,4-dione (**1**) with hydrazine (**2a**) under various conditions [2–4] and by dipolar cycloaddition of 2-diazo-1,1,1-trifluoroethane with 3,3,3-trifluoropropyne [5]. Synthesis of 1-aryl and 1-aroyl-3,5-bis(trifluoromethyl)pyrazoles **4b–e** has hitherto only been claimed by Claire *et al.* [2] by reaction of substituted hydrazines **2b–e** with diketone **1** in boiling ethanol. Since it is difficult to rationalise the spectroscopic data reported [2] with the aromatic pyrazole claimed, and in view of our observations [1] that treatment of heteroarylhydrazines with 1,1,1-trifluoropentane-2,4-dione sometimes affords only the partial condensation products, i.e. the 5-hydroxy-4,5-dihydropyrazoles, a re-investigation of the reaction of **1** with hydrazines **2a–e** was undertaken.

Results and discussion

Treatment of 1,1,1,5,5,5-hexafluoropentane-2,4-dione (**1**) with an equimolar amount of hydrazine hydrate in refluxing ethanol gave, as predicted, 3,5-bis(trifluoromethyl)pyrazole (**4a**) as volatile white crystals with a distinctive odour. The ^{1}H NMR spectrum was consistent with an aromatic pyrazole structure. The 4-H atom gives a resonance at δ 6.95, which is slightly broadened by coupling to ^{19}F. Only one ^{19}F NMR signal was evident for **4a**, owing to rapid site exchange of the NH proton. The electron-impact mass spectrum of this pyrazole showed an abundant molecular ion at *m/z* 204, with no evidence of ions at higher mass (e.g. *m/z* 218 for the hydroxydihydropyrazole structure).

However, the ^{1}H NMR spectra of the heterocycles formed from **1** and the substituted hydrazines **2b–e** showed no signal in the region expected for the 4-H atom of an aromatic pyrazole (δ 5.8–δ 7.2) [1, 6]. Claire *et al.* [2] also report no signals in this region but claim that 4-H resonates at $\delta \sim 3.6$; no integral or multiplicity data were given. The extensive study by Tensmeyer and Ainsworth [6] of substituent effects on the chemical shifts in pyrazoles does not support this assignment. In our work, the signals in the region δ 3–δ 4 comprised two doublets, the integral of each doublet corresponding to one proton. The coupling constants between these doublets were >14 Hz, typical values for geminal coupling. Thus these signals can be assigned to a prochiral CH_2 group in an asymmetric environment. Broad resonances due to OH were also observed. On the basis of these data, the hydroxydihydropyrazole structures **3b–e** are proposed, rather than the pyrazoles **4b–e** claimed [2].

The electron-impact (EI) mass spectra of the 5-hydroxydihydropyrazoles **3c–e** at 70 eV ionisation energy revealed abundant molecular ions. The molecular ion of **3b** was present in the EI spectrum only at 1% abundance, but abundant ions were observed at *m/z* 326 (M) in the chemical ionisation (CI) spectrum and at *m/z* 327 (M+H) in the positive ion fast atom bom-

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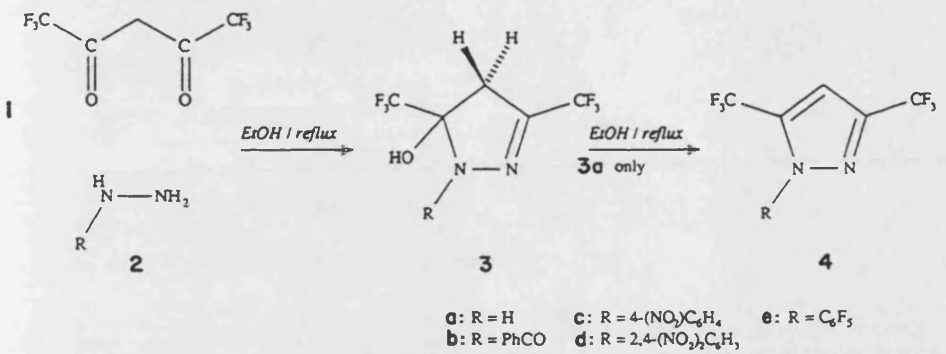

Scheme 1. Reaction of hydrazines with 1,1,1,5,5,5-hexafluoropentane-2,4-dione in boiling ethanol.

bardment (FAB) spectrum. Dehydration was not favoured (giving M-18) but loss of $\cdot CF_3$ gave highly abundant peaks at (M − 69) in all cases. The reported interpretation [2] of similar data, representing the ions at highest mass as molecular clusters of (pyrazole + H_2O), should be regarded as unlikely under EI conditions.

Experimental

^1H NMR spectra were obtained at 270 MHz and 400 MHz using JEOL GX270 and JEOL EX400 spectrometers, respectively (solvent, $CDCl_3$; internal standard, $SiMe_4$). The ^{19}F NMR spectrum was obtained at 84.25 MHz using a JEOL FX90Q spectrometer (solvent, H_2O/phosphate buffer pH 7.3; external standard, $NaPF_6$ in phosphate buffer pH 7.3). Low-resolution EI, CI (isobutane) and FAB and high-resolution EI mass spectra were furnished by a VG 7070 spectrometer, whereas a ZAB-E instrument gave the high-resolution FAB spectrum. Solvents were evaporated under reduced pressure, except where noted.

3,5-Bis(trifluoromethyl)pyrazole (4a)

1,1,1,5,5,5-Hexafluoropentane-2,4-dione (**1**) (4.16 g, 20 mmol) was added to hydrazine hydrate (**2a**) (1.2 g, 24 mmol) in ethanol (50 ml). The mixture was boiled under reflux for 18 h and allowed to cool. The solvent was distilled off carefully at atmospheric pressure. The residue was distilled at atmospheric pressure. The distillate, in dichloromethane, was dried (anhydrous $MgSO_4$) and filtered and the solvent evaporated to give **4a** (1.04 g, 25%) as white crystals, m.p. 69–70 °C (lit. [2] m.p. 71–72 °C). ^1H NMR (270 MHz) δ: 6.95 (br s, 1H, 4-H); 11.97 (br, 1H, NH) ppm. ^{19}F NMR δ: +10.92 (s, 6F, $2\times CF_3$) ppm. MS (EI) *m/z*: 204 (M); 185; 154; 69.

1-Benzoyl-3,5-bis(trifluoromethyl)-4,5-dihydro-5-hydroxypyrazole (3b)

1,1,1,5,5,5-Hexafluoropentane-2,4-dione (**1**) (300 mg, 1.44 mmol) was added to benzoyl hydrazide (**2b**) (136 mg, 1 mmol) in ethanol (50 ml). The mixture was boiled under reflux for 5 h and allowed to cool. The solvent was evaporated to give **3b** (315 mg, 96%) as a white solid, m.p. 82–84 °C (lit. [2] m.p. 84–85 °C claimed for pyrazole **4b**). ^1H NMR (270 MHz) δ: 3.38 (d septet, 1H, $J_{H-H}=19.5$ Hz, $J_{H-F}=1.5$ Hz, pyrazole 4-H); 3.56 (br d, 1H, $J_{H-H}=19.5$ Hz, pyrazole 4-H); 6.43 (br s, 1H, OH); 7.47 (*c.* t, 2H, $J=7.5$ Hz, Ar 3,5-H_2); 7.59 (tt, 1H, $J=7.5$ Hz, $J=1.3$ Hz, Ar 4-H); 7.86 (*c.* d, 2H, $J=7.5$ Hz, Ar 2,6-H_2) ppm. MS (EI) *m/z*: 326 (M, 1%); 105 (PhCO, 100%). MS (CI) *m/z*: 326 (M). MS (FAB; +ve ion) *m/z*: 327.0599 (M + H) ($C_{12}H_9F_6N_2O_2$ requires: 327.0568).

3,5-Bis(trifluoromethyl)-4,5-dihydro-5-hydroxy-1-(4-nitrophenyl)pyrazole (3c)

The dione **1** (300 mg, 1.44 mmol) was added to 4-nitrophenylhydrazine (**2c**) (200 mg, 1.3 mmol) in ethanol (50 ml). The mixture was boiled under reflux for 3 h and allowed to cool. The solvent was evaporated to give **3c** (410 mg, 92%) as a yellow solid, m.p. 105–106 °C (lit. [2] m.p. 105–108 °C claimed for pyrazole **4c**). ^1H NMR (270 MHz) δ: 3.42 (d septet, 1H, $J_{H-H}=19.6$ Hz, $J_{H-F}=1.5$ Hz, pyrazole 4-H); 3.74 (br d, 1H, $J_{H-H}=19.6$ Hz, pyrazole 4-H); 5.75 (br, 1H, OH); 7.60 (d, 2H, $J=9.4$ Hz, Ar 2,6-H_2); 8.12 (d, 2H, $J=9.4$ Hz, Ar 3,5-H_2) ppm. MS (EI) *m/z*: 343 (M); 274 (100%); 228. Analysis: Found: C, 38.70; H, 2.03; N, 12.50%. $C_{11}H_7F_6N_3O_3$ requires: C, 38.48; H, 2.06; N, 12.25%.

3,5-Bis(trifluoromethyl)-4,5-dihydro-1-(2,4-dinitrophenyl)-5-hydroxypyrazole (3d)

The dione **1** (208 mg, 1.0 mmol) was added to 2,4-dinitrophenylhydrazine (**2d**) (260 mg, 1.3 mmol) in ethanol (60 ml). The mixture was boiled under reflux for 5 h and allowed to cool. Evaporation of the solvent gave a gum which was extracted with methanol. Evap-

oration of the methanol gave **3d** (300 mg, 77%) as a viscous orange oil which crystallised on standing to an orange–yellow solid, m.p. 81–83 °C (lit. [2] m.p. 82–83 °C claimed for pyrazole **4d**) ^{1}H NMR (400 MHz) δ: 3.06 (d, 1H, J=14.6 Hz, pyrazole 4-H); 3.18 (d, 1H, J=14.6 Hz, pyrazole 4-H); 5.1 (br, 1H, OH); 8.04 (d, 1H, J=9.5 Hz, Ar 6-H); 8.40 (dd, 1H, J=9.5 Hz, J=2.4 Hz, Ar 5-H); 9.14 (d, 1H, J=2.4 Hz, Ar-3-H) ppm. MS, (EI) *m/z*: 388.0179 (M) ($C_{11}H_6F_6N_4O_5$ requires: 388.0242); 291.0327 ($M-CF_3CO\cdot$) ($C_9H_6F_3N_4O_4$ requires: 291.0341).

3,5-Bis(trifluoromethyl)-4,5-dihydro-5-hydroxy-1-(pentafluorophenyl)pyrazole (3e)

The dione **1** (300 mg, 1.44 mmol) was added to pentafluorophenylhydrazine (**2e**) (198 mg, 1.0 mmol) in ethanol (50 ml). The mixture was boiled under reflux for 5 h and allowed to cool. The solvent was evaporated to give **3e** (355 mg, 91%) as colourless prisms, m.p. 61–62 °C (lit. [2] m.p. 60–62 °C claimed for pyrazole **4e**). ^{1}H NMR (400 MHz) δ: 3.32 (br d, 1H, J=18.9 Hz, pyrazole 4-H); 3.59 (dq, 1H, J_{H-H}=18.9 Hz, J_{H-F}=1.5 Hz, pyrazole 4-H); 6.13 (br s, 1H, OH) ppm. MS (EI) *m/z*: 388.0099 (M) ($C_{11}H_3F_{11}N_2O$ requires: 388.0070); 319 (100%); 299.

Conclusions

Whereas condensation of hydrazine (**2a**) with 1,1,1,5,5,5-hexafluoropentane-2,4-dione (**1**) in boiling ethanol gives the aromatic pyrazole **4a**, treatment of this dione with more hindered and less nucleophilic aryl- and acyl-hydrazines gives only the partial condensation products, the 4,5-dihydro-5-hydroxypyrazoles **3b–e**. A previous report [2] of the formation of the 1-substituted pyrazoles **4b–e** must be regarded as erroneous. The conditions required to effect the dehydration **3b–e**→**4b–e** are under investigation.

Acknowledgements

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PUBLICATION 59

Poly(ADP-Ribosylation) as Target for Cancer Chemotherapy

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[Review]

Poly(ADP-ribosylation) as target for cancer chemotherapy

The enzyme poly(ADP-ribose) polymerase (ADPRP), which is abundant in all cell nuclei, is an important regulatory enzyme. Stimulated by DNA strand breaks it links poly(ADP-ribose) chains to nuclear proteins, and to DNA repair enzymes including ADPRP itself. Inhibition of this enzyme impedes DNA repair and probably interferes with the vital processes of differentiation, gene expression, DNA replication, and genetic recombination.[1,2] The high levels of ADPRP observed in certain tumour cells suggest this enzyme as a target for chemotherapy,[3] and it has been argued that inhibition of ADPRP could have a role in the treatment of retroviral infection by affecting processes such as integration of viral sequences into DNA and reverse transcription.[4] However, the main focus of interest in ADPRP inhibitors has been in exploiting their ability to inhibit DNA repair, in combination with DNA-damaging treatments such as radiotherapy and alkylating agents.

Early attempts focused on analogues of NAD^+, from which the poly(ADP-ribose) derives. Molecular mimics of adenosine would not be much use because they would also bind to ADP and ATP requiring enzymes. However, nicotinamide mimics should show selectivity, and 3-aminobenzamide (3-AB) was identified in 1980 as an ADPRP inhibitor[5] and became a standard laboratory tool for inhibition of DNA repair. Inhibition of ADPRP might also exert a more direct antitumour effect. For example, some inhibitors (1,2-benzopyrone [coumarin], benzamide, hexamethylene bisacetamide) inhibit *ras*-activated cells in vitro and suppress their tumorigenicity in vivo.[6] These agents seem to bind to the DNA interactive site on the enzyme.

Modulation or inhibition of DNA repair may explain the efficacy of drugs used in combination with cisplatin, including 5-fluorouracil, cytarabine, and hydroxyurea.[7] Refinement of the structural requirements for potent inhibition of ADPRP opens up the realistic prospect of clinically useful chemosensitising or radiosensitising drugs acting via inhibition of DNA repair.[8,9] Hitherto, the chemists' approach has been limited to substrate analogues acting as competitive inhibitors; now effort should be directed towards the design of more potent agents, including the so-called "suicide" (irreversible) inhibitors. Selectivity for target should be feasible too for we already know that several ADPRP inhibitors are orders of magnitude less inhibitory towards the mono(ADP-ribosyl)ation involved in the control of other enzymes.[9] Whether such drugs will display selectivity for cancer cells is less certain, but one approach to increase selectivity is to exploit the lower oxygen concentration in the core of solid tumours by designing pro-drugs which will be metabolically activated to inhibitors of ADPRP in this environment. Prospects for enhancing the efficacy of chemotherapy and radiotherapy via inhibition of ADPRP, and hence DNA repair, look excellent but much remains to be done.

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PUBLICATION 60

Tumour-Targetted Boranes. Part 2. Coupling of *Closo*carboranes to Substituted 2-Nitroimidazoles *via* 1,3-Dipolar Cycloaddition

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Journal of the Chemical Society, Perkin Transactions 1, **1994**, 203-210.

Tumour-targetted Boranes. Part 2.[1] Coupling of *closo*-Carboranes to Substituted 2-Nitroimidazoles *via* 1,3-Dipolar Cycloaddition

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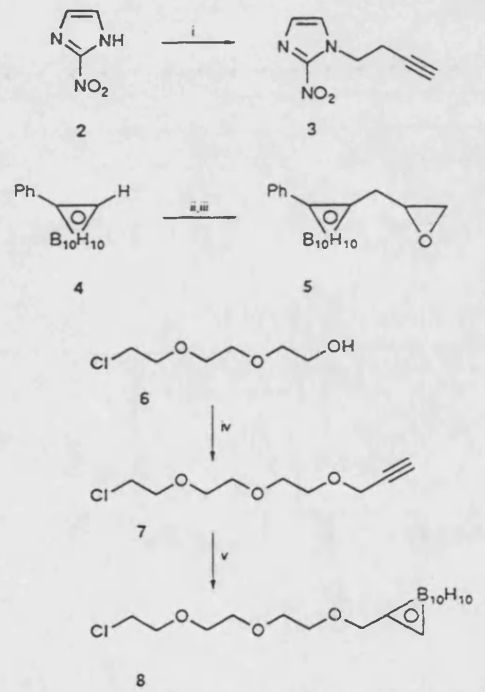
Carboranes targetted to specific tumour tissues are important for boron neutron capture therapy of cancer. Direct syntheses of carboranes linked to 2-nitroimidazole were unsuccessful. A mild procedure for 1,3-dipolar cycloaddition of 4-(carboranylmethoxy)benzonitrile *N*-oxide **32** with a nitroimidazolyl-alkene **27** and with nitroimidazolyl-alkynes **3** and **30** has been developed, using a series of model reactions, yielding a dihydroisoxazole **28** and the isoxazoles **29** and **31**, respectively. The nitrile oxide **32** is unusually stable. Dithioacetals are shown to be suitable protecting groups for aromatic aldehydes under the vigorous reductive and Lewis acidic-basic conditions of carborane formation. 6-Methoxy-4*H*-[1]benzopyrano[4,3-*c*]isoxazole **16** has been synthesised by intramolecular 1,3-dipolar cycloaddition. The structure of the isoxazole derivative **29** has been confirmed by an X-ray crystal structure analysis.

Boron neutron capture therapy (BNCT) is of increasing interest as a strategy for treatment of various cancers, notably gliomas and melanomas.[2] When the ^{10}B isotope is irradiated with slow ('thermal') neutrons, an [n,α] reaction ensues, giving mainly ^{7}Li and ^{4}He nuclei, along with kinetic energy (2.31 MeV). With this energy, the ^{4}He has a range of *ca.* 9 μm in tissue (*ca.* 1 cell diam.). Thus, damage is limited to the cell containing the boron and to adjacent cells. Early studies of BNCT gave mixed results.[3] Failures were attributed to inadequate concentrations of ^{10}B in the tumour tissue or to lack of selectivity of disposition of ^{10}B, leading to damage to normal tissue. Recently, carboranes have been linked to nucleosides,[4] to amino acids[5] and to porphyrins[6] in attempts to target boron to tumours. 1-Substituted 2-nitroimidazoles are known to be selectively retained in poorly vascularised hypoxic tumour tissue by reductive metabolism to electrophiles.[7] As part of a programme of synthesis and evaluation of nitroimidazoles in the treatment of cancer,[1,8-10] we propose that compounds containing 10–12 boron atoms linked to 2-nitroimidazole would form a useful method of concentrating boron in solid tumours. Derivatives of 1,2-dicarba-*closo*-dodecaborane(12)[11] ('carborane', **1**, Fig. 1) were selected for linkage to 2-nitroimidazole in view of their good chemical stability relative to other boron clusters and their predicted metabolic inertness. Before our preliminary communication,[1] no report of nitroimidazoles bearing boron had been made in the journal literature.

In most cases, carboranes are readily formed[11] by reaction of alkynes and decaborane(14) ($B_{10}H_{14}$) in the presence of boiling Lewis bases for long reaction times. Consequently, the nitroimidazolylalkyne **3** was prepared by alkylation of 2-nitroimidazole **2** with but-3-ynyl tosylate. However, treatment of **3** with decaborane(14) under the standard reaction conditions (acetonitrile, heat) gave only an inseparable mixture of materials probably arising from reduction of the nitroimidazole. Such reduction is unsurprising, since simple and complex boranes are widely used as reducing agents but 1-substituted 2-nitroimidazoles are themselves known to be readily reduced (E^{1}_{7} = −389 mV)[10]. Thus, the carborane must be formed before the nitroimidazole is introduced into the molecule.

2-Nitroimidazole reacts under basic conditions with oxiranes to give substituted nitroimidazolylethanols[8] and the anion of **2** can be alkylated[10] at elevated temperatures (> 130 °C) by a variety of halogenoalkanes and alkyl tosylates. Carboranes bearing both types of electrophile were assembled for investigation of their reaction with **2**. Treatment of phenylethyne with decaborane(14) gave the known[11] monosubstituted carborane **4** and treatment of the corresponding anion with epichlorohydrin gave the carboranylmethyloxirane **5** in good yield. The



Scheme 1 Syntheses of substituted nitroimidazole **3** and substituted carboranes **4**, **5** and **8**, precursors for attempts at 'direct' routes to nitroimidazole-carboranes: i, $HC{\equiv}CCH_2CH_2OTs$–$KOBu^t$; ii, BuLi; iii, epichlorohydrin; iv, NaH-$HC{\equiv}CCH_2Br$; v, $B_{10}H_{14}$, MeCN

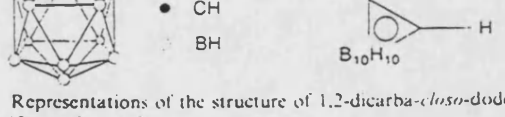
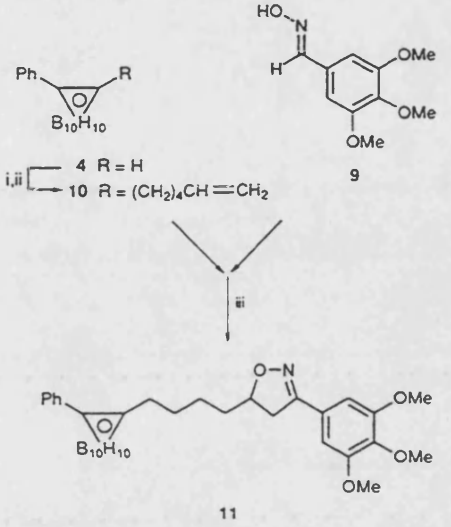


Fig. 1 Representations of the structure of 1,2-dicarba-*closo*-dodecaborane(12) ('carborane') **1**

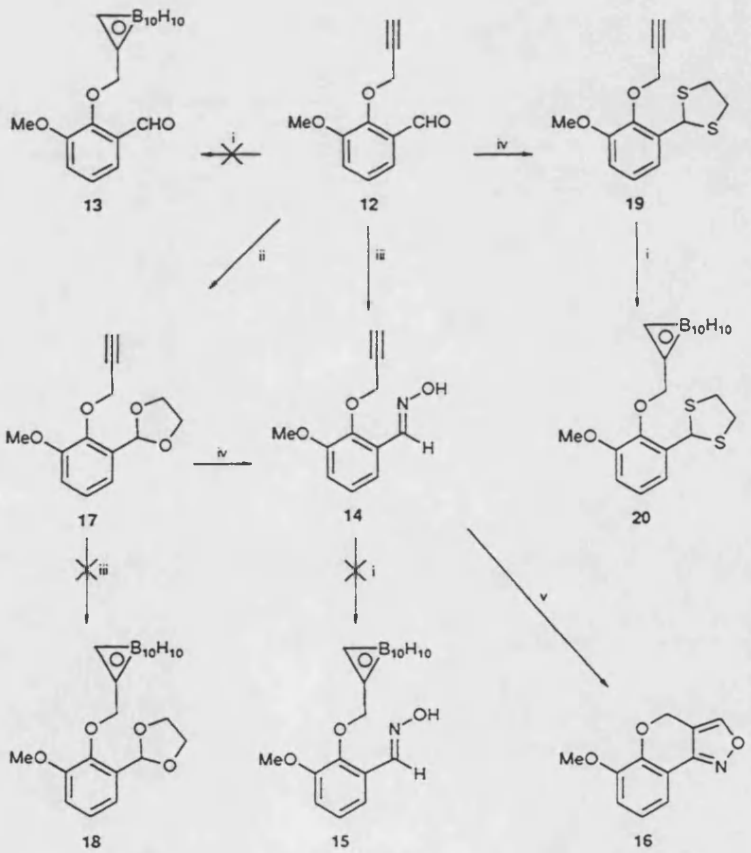
chloro alcohol **6** was alkylated using the reactive electrophile prop-2-ynyl bromide, giving the chloroalkyne **7**. The corresponding carborane **8** was formed in satisfactory yield. Neither **5** nor **8** reacted with 2-nitroimidazole. Similarly, the lithio derivative of **4** did not react with 2-nitro-1-(oxiranylmethyl)imidazole.[8]

Clearly, the conditions required for the formation of nitroimidazolylcarboranes by these 'direct' methods were too harsh to permit the co-existence of the nitroimidazole and the carborane. 1,3-Dipolar cycloadditions of nitrile oxides with alkenes and alkynes proceed[12] under mild conditions; this method of carbon–carbon bond formation was investigated as a route to the target compounds. Nitrile oxides are most conveniently formed by oxidation of the corresponding aldoximes. The series of model reactions shown in Schemes 2 and 3 were studied to check the compatibility of these oxidative conditions (aqueous sodium hypochlorite) with carboranes and the stability of the precursor oximes and synthons thereof to decaborane(14). Treatment of 3,4,5-trimethoxybenzaldehyde oxime **9** with aqueous sodium hypochlorite gave the nitrile oxide which reacted *in situ* with alkenylcarborane **10** (prepared by alkylation of the anion of **4**) to give the dihydroisoxazole **11**. Thus, the stability of carboranes to sodium hypochlorite is demonstrated.

To study the effects of the carborane formation conditions on aldehyde and oxime groups and their synthetic equivalents, 3-methoxy-2-prop-2-ynyloxybenzaldehyde **12** was converted into its oxime **14**, as shown in Scheme 3. However, both aldehyde and oxime functions were found to be degraded by decaborane(14), preventing the synthesis of the model carboranyl aldehyde **13** and the model carboranyl oxime **15** by these direct routes. Interestingly, the intramolecular 1,3-dipolar cycloaddition of the nitrile oxide derived from **14** was successful in giving the 4*H*-[1]benzopyrano[4,3-*c*]isoxazole **16**. The yield was modest, presumably owing to the usually unfavourable regiochemistry of cycloaddition[12] being forced by the geometry



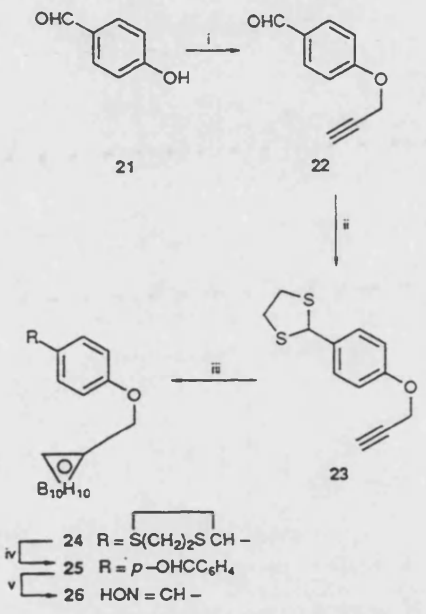
Scheme 2 Model reactions to demonstrate the compatibility of carborane with the conditions required for 1,3-dipolar cycloaddition: i, BuLi; ii, $H_2C{=}CH(CH_2)_4Br$; iii, $NaOCl$-H_2O-CH_2Cl_2



Scheme 3 Model reactions to investigate the stability of aldehydes and their protected forms to $B_{10}H_{14}$ and synthesis of methoxybenzopyranoisoxazole **16**: i, $B_{10}H_{14}$-MeCN; ii, $HOCH_2CH_2OH$-TsOH; iii, $HONH_2$-HCl-Na_2CO_3; iv, $HSCH_2CH_2SH$-BF_3-Et_2O; v, NaOCl

of the nitrile oxide; this heterocyclic system has hitherto been reported only once.[13] The cyclic acetal **17**, formed from **12** by treatment with ethane-1,2-diol under acidic conditions, was also not stable to decaborane(14). Protection of the aldehyde as the cyclic dithioacetal **19** by Lewis acid-catalysed reaction with ethane-1,2-dithiol was, however, effective in permitting the synthesis of the carborane **20**. Hence, aldehydes can be protected as dithioacetals during formation of carboranes. Assembly of nitroimidazolylcarboranes was, therefore, expected to be feasible by a route involving an appropriate 1,3-dipolar cycloaddition.

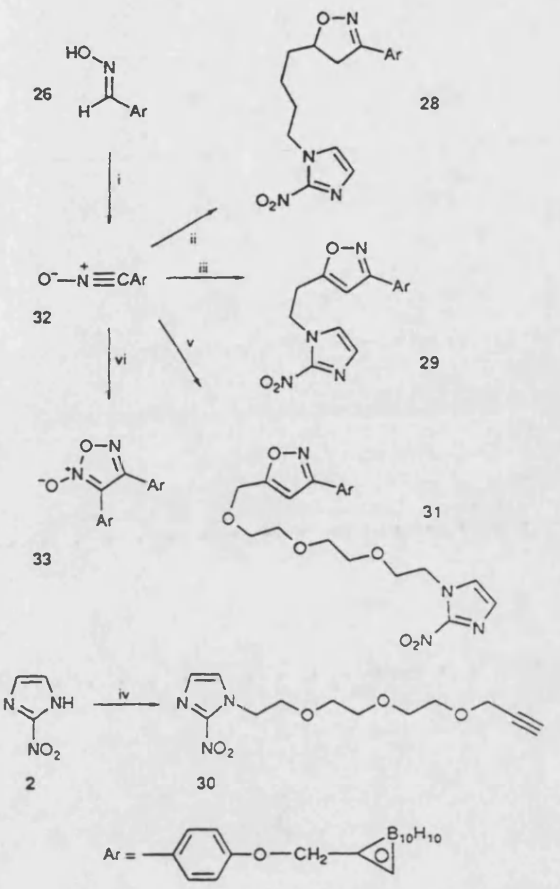
To provide the central framework to support carborane and oxime groups, 4-hydroxybenzaldehyde **21** was prop-2-ynylated by the method of Matolcsy *et al.*,[14] giving the alkynealdehyde **22** (Scheme 4). Protection of the aldehyde as the cyclic dithio-



Scheme 4 Synthesis of the carborane-aldoxime **26**, a precursor of a carborane–nitrile oxide: i, NaH–HC≡CCH_2Br; ii, $HSCH_2CH_2SH$–BF_3·Et_2O; iii, $B_{10}H_{14}$–MeCN; iv, $Hg(ClO_4)_2$; v, $HONH_2$·HCl–Na_2CO_3

acetal **23** was achieved in 80% yield. As predicted by the model experiments, this protecting group resisted prolonged treatment with decarborane(14) in boiling acetonitrile, which furnished the carborane **24**. Deprotection was effected virtually quantitatively within 5 min using mercury(II) perchlorate to give the carboranyl aldehyde **25** which was converted into the carboranyl oxime **26**. This oxime represents the precursor to the nitrile oxide **32** for 1,3-dipolar cycloaddition with the range of nitroimidazolyl-alkenes and -alkynes (Scheme 5). The polyether-linked nitroimidazole **30** was synthesised by reaction of the potassium salt of 2-nitroimidazole with the alkyne **7** under the usual vigorous conditions required for alkylation of this weak nucleophile (dimethylformamide, 130 °C).

Oxidation/elimination of the carboranyl oxime **26** using aqueous sodium hypochlorite to give the intermediate nitrile oxide **32** was very rapid as shown by TLC but prolonged reaction times at ambient temperature were required for cycloaddition *in situ* with nitroimidazolylalkene **27**[10] and nitroimidazolylalkynes **3** and **30**. The dihydroisoxazole-linked compound **28** and the isoxazole-linked compounds **29** and **31**, respectively, in which both 2-nitroimidazole and carborane moieties are present, were formed in excellent yields based on consumption of starting materials (Scheme 5).



Scheme 5 Dipolar cycloaddition reactions of carborane-nitrile oxide **32** and synthesis of nitroimidazole-carboranes **28**, **29** and **31**: i, NaOCl; ii, 1-hex-5-enyl-2-nitroimidazole **27**; iii, nitroimidazole-alkyne **3**; iv, KOBu^t–alkyne **7**; v, nitroimidazole-alkyne **30**; vi, toluene, reflux

In each case, even after reaction for several days, no boron-containing compounds other than **26**, **28**, **29**, **31** and **32** were evident in the reaction mixtures and it was possible to isolate the unchanged nitrile oxide **32** by chromatography. This nitrile oxide is remarkably stable, with little decomposition on storage for several weeks at ambient temperature. In contrast, the half-life of most aromatic nitrile oxides is reported[12] to be only a few hours. It did not react with cyclohexene, a weak dipolarophile,[12] at reflux temperature and was converted into its dimer, the 1,2,5-oxadiazole 2-oxide **33**, only when heated in boiling toluene.

Additional evidence for the structure of target compound **29** was provided by a determination of the crystal structure by X-ray diffraction methods. Recrystallisation of a sample of compound **29** from benzene afforded a crystal which was not of high quality but which diffracted strongly and was, therefore, adequate for this determination. Other solvents gave only microcrystalline material. The crystal had approximate dimensions 0.4 × 0.4 × 0.2 mm.

Crystal Data.—$C_{17}H_{24}B_{10}N_4O_4$·$\frac{1}{2}C_6H_6$, M = 495.5, triclinic, a = 7.320(2), b = 11.275(3), c = 16.359(4) Å, α = 94.86(4), β = 92.83(3), γ = 100.78(4), U = 1318.6 Å^3, space group $P\bar{1}$, Z = 2, D_c = 1.25 g cm^{-3}, μ(Mo-K_α) = 0.47 cm^{-1}, F(000) = 514. Data were measured at ambient temperature on a Hilger and Watts Y290 four-circle diffractometer in the range $2 \leqslant \theta \leqslant 22°$. 3444 Reflections were collected, of which 1430 were unique with $I \geqslant 3\sigma(I)$. Data were corrected for Lorentz and polarisation effects but not for absorption. The structure

was solved by direct methods and was refined using the SHELX[15] suite of programmes. In the final least-squares cycles, all the atoms were allowed to vibrate anisotropically. The half portion of benzene solvent molecule which accompanies the structure is proximate to a centre of symmetry. This generates the remaining atoms to complete the benzene ring. Hydrogen atoms were included at calculated positions where appropriate, except in the solvent species. In the later stages of refinement, the molecule and associated solvent of recrystallisation were treated as separate blocks. Final residuals after eight cycles of least squares were $R = R_w = 0.1218$. Maximum final shift/esd was 0.009. The maximum and minimum residual densities were 0.12 and -0.10 eÅ^{-3}, respectively.

Table 1 gives selected bond lengths, Table 2 gives selected bond angles and Table 3 gives selected torsion angles for the structure of the substituted nitroimidazolyl-carborane **29**. Full tables of final fractional coordinates and isothermal parameters, bond distances, bond angles, torsion angles and anisotropic temperature factors have been deposited with the Cambridge Crystallographic Data Centre.* The asymmetric unit is shown in Fig. 2, along with the labelling scheme used. In the crystal, the compound adopts an extended conformation, with torsion angles of the two flexible units linking the nitroimidazole to the isoxazole and the benzene to the carborane [N(1)–C(4)–C(5)–C(6) and C(12)–O(4)–C(15)–C(16), respectively] being close to 180°. The tilt angle between the two adjacent aromatic rings, the benzene and the isoxazole, is 11.4°, *i.e.* the two rings diverge only slightly from coplanarity. The carborane forms a slightly distorted icosahedron, as reported by the bond lengths and bond angles to C(16) and C(17). Hence, this carborane corresponds to the usual icosahedral shape for such C_2B_{10} structures (*e.g.* ref. 16), rather than the 'basket handle' structure proposed by Zakharkin *et al.*[17]

Table 1 Selected bond lengths for the crystal structure of the nitroimidazole-carborane **29**

Bond	Bond length (Å)	Bond	Bond length (Å)
O(1)–N(3)	1.20(3)	O(2)–N(3)	1.24(2)
O(3)–N(4)	1.48(2)	O(3)–C(6)	1.29(2)
O(4)–C(12)	1.42(2)	O(4)–C(15)	1.35(3)
N(3)–C(1)	1.42(2)	N(1)–C(1)	1.38(2)
N(2)–C(1)	1.30(3)	N(1)–C(3)	1.28(2)
N(2)–C(2)	1.37(2)	C(2)–C(3)	1.44(3)
C(15)–C(16)	1.63(3)	C(16)–C(17)	1.61(3)
C(16)–B(1)	1.72(3)	C(16)–B(2)	1.51(3)
C(16)–B(3)	1.62(3)	C(16)–B(4)	1.73(3)
C(17)–B(1)	1.67(3)	C(17)–B(2)	1.67(3)
C(17)–B(5)	1.66(3)	C(17)–B(6)	1.66(3)
B(1)–B(4)	1.85(3)	B(1)–B(5)	1.80(3)
B(1)–B(9)	1.85(4)	B(2)–B(3)	1.75(3)
B(2)–B(6)	1.81(3)	B(2)–B(7)	1.79(4)
B(3)–B(4)	1.61(3)	B(3)–B(7)	1.85(3)
B(3)–B(8)	1.78(3)	B(4)–B(8)	1.66(4)
B(4)–B(9)	1.85(3)	B(5)–B(6)	1.59(4)
B(5)–B(9)	1.72(3)	B(5)–B(10)	1.65(4)
B(6)–B(7)	1.91(4)	B(6)–B(10)	1.69(4)
B(7)–B(8)	1.86(3)	B(7)–B(10	1.95(3)
B(8)–B(9)	1.63(4)	B(8)–B(10)	1.66(3)
B(9)–B(10)	1.57(3)		

Table 2 Selected bond angles for the crystal structure of the nitroimidazole-carborane **29**

Atoms	Bond angle (°)	Atoms	Bond angle (°)
N(3)–C(1)–N(1)	123(2)	N(3)–C(1)–N(2)	125(2)
N(1)–C(1)–N(2)	112(1)	C(1)–N(3)–O(1)	121(2)
C(1)–N(3)–O(2)	114(2)	O(1)–N(3)–O(2)	124(2)
C(1)–N(1)–C(4)	129(1)	C(3)–N(1)–C(4)	124(1)
C(5)–C(4)–N(1)	107(1)	C(4)–C(5)–C(6)	105(1)
C(5)–C(6)–O(3)	116(1)	C(5)–C(6)–C(7)	130(1)
C(8)–C(9)–C(10)	117(1)	C(10)–C(9)–C(14)	122(2)
C(11)–C(12)–C(13)	126(2)	O(4)–C(15)–C(16)	111(1)
C(15)–C(16)–C(17)	113(2)	C(15)–C(16)–B(1)	111(1)
C(15)–C(16)–B(2)	117(1)	C(15)–C(16)–B(3)	122(1)
C(15)–C(16)–B(4)	122(1)		

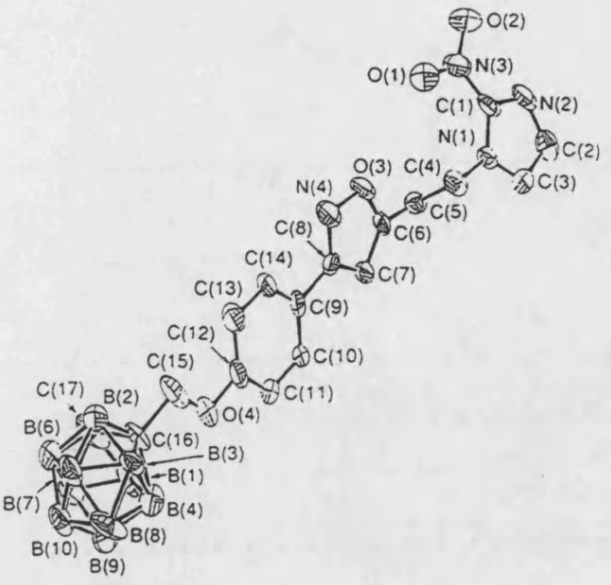


Fig. 2 Asymmetric unit of the crystal structure of the nitroimidazole-carborane **29** with the labelling scheme used for the crystallographic structure determination

The oxidising character of 2-nitroimidazoles and the potential reductant activity of boron hydrides preclude the formation of linked nitroimidazolyl-carboranes by the vigorous conditions required for 'direct' methods, such as treatment of a nitroimidazolyl-alkyne with decaborane(14) or alkylation of the anion of 2-nitroimidazole with electrophiles linked to carboranes. Model reactions have demonstrated that both 2-nitroimidazole and carborane moieties are stable to the conditions of the generation of nitrile oxides and their 1,3-dipolar cycloaddition to alkenes and alkynes. These model studies have also shown the utility and importance of dithioacetal protection for aromatic aldehydes under the conditions of formation of carboranes. On the basis of these studies, the unusually stable carboranylmethoxyphenyl nitrile

* For details see Instructions for Authors, *J. Chem. Soc., Perkin Trans. 1*, 1994, Issue 1.

Table 3 Selected torsion angles for the crystal structure of nitroimidazole-carborane **29**

Atoms	Torsion angle (°)	Atoms	Torsion angle (°)
O(1)–N(3)–C(1)–N(1)	10.5	N(1)–C(4)–C(5)–C(6)	174.9
N(4)–C(8)–C(9)–C(14)	11.4	C(12)–O(4)–C(15)–C(16)	171.4

oxide **32** was synthesised and was shown to react efficiently with nitroimidazolyl-alkenes and -alkynes to give the required linked nitroimidazole-carboranes **28**, **29** and **31**.

1,3-Dipolar cycloaddition of nitrile oxides is, therefore, demonstrated to be a mild process which has great potential in medicinal chemistry for joining chemically sensitive targetting moieties to pharmacophores. A new carbon–carbon bond is formed and the linking (dihydro)isoxazole gives opportunity for further elaboration. Unfortunately, the target compounds **28**, **29** and **31** were found to be insufficiently soluble in water to permit satisfactory biological evaluation. The contruction of alternative, more polar links between nitroimidazole and carborane is under active investigation.

Experimental

JEOL GX270 and EX400 instruments furnished the NMR spectra. $CDCl_3$ was the solvent for NMR spectroscopy unless otherwise noted. The external reference for ^{11}B NMR was boron trifluoride–diethyl ether complex. *J* Values are recorded in Hz. Solutions in organic solvents were dried with anhydrous magnesium sulfate. Solvents were evaporated under reduced pressure. The chromatographic stationary phase was silica gel. Distillation was carried out using a Büchi Kugelrohr apparatus; b.p.s refer to the temperature of the oven. 1-(Hex-5-enyl)-2-nitroimidazole **27** was prepared as described previously by us.[10] DMF refers to dimethylformamide; THF refers to tetrahydrofuran. M.p.s are uncorrected.

1-But-3-ynyl-2-nitroimidazole **3**.—2-Nitroimidazole **2** (340 mg, 3 mmol) in dry DMF (10 cm^3) was treated with potassium *tert*-butoxide (340 mg, 3 mmol) at 130 °C until it dissolved and then for a further 10 min. The mixture was cooled to 80 °C, but-3-ynyl-4-methylbenzenesulfonate[18] (670 mg, 3 mmol) and sodium iodide (10 mg) were added to it and the whole heated at 130 °C for 10 min. Evaporation of the solvent from the mixture left a residue which was dissolved in water (5 cm^3) and the solution was filtered and extracted with chloroform. The extract was evaporated to give a yellow semi-solid which was recrystallised from pentane–benzene (CAUTION) to afford the *title compound* **3** (290 mg, 59%) as pale yellow crystals, m.p. 71–72 °C (Found: C, 50.8; H, 4.2; N, 25.8. $C_7H_7N_3O_2$ requires C, 50.9; H, 4.25; N, 25.45%); δ 2.08 (1 H, t, *J* 2.5, C≡CH), 2.81 (2 H, dt, *J* 2.5 and 6.4, $CH_2C{\equiv}C$), 4.58 (2 H, t, *J* 6.4, imidazole-CH_2) and 7.17 (1 H, br s) and 7.24 (1 H, br s) (imidazole 4,5-H).

[(2-Phenyl-1,2-dicarba-closo-dodecaboran(12)-1-yl)methyl]-oxirane **5**.—Butyllithium (1.6 mol dm^{-3} in hexanes; 3.1 cm^3, 5 mmol) was added to 1-phenyl-1,2-dicarba-*closo*-dodecaborane(12) **4**[11] (880 mg, 4 mmol) in dry THF (20 cm^3) at −78 °C. The mixture was stirred at −78 °C for 30 min after which chloromethyloxirane (460 mg, 5 mmol) was added to it. The mixture was then allowed to warm to 20 °C during 16 h after which it was partitioned between water and dichloromethane. The organic phase was separated and evaporated and the residue was chromatographed (pentane–dichloromethane, 1:1) to give the *title compound* **5** (850 mg, 62%) as a white solid, m.p. 63–65 °C (Found: C, 47.7; H, 7.35. $C_{11}H_{20}B_{10}O$ requires C, 47.8; H, 7.3%); δ_H 1.7–3.1 (10 H, br m, $B_{10}H_{10}$), 1.90 (1 H, dd, *J* 15.4 and 5.9) and 2.08 (1 H, dd, *J* 15.4 and 6.1) (carborane-CH_2), 2.17 (1 H, dd, *J* 4.9 and 2.4) and 2.71 (1 H, dd, *J* 4.6 and 4.1) (oxirane 2-H_2), 2.97 (1 H, m, oxirane 1-H) and 7.45–7.65 (5 H, m, Ph-H_5); δ_B −15.5 to −12.5 (8 B, br) and −7.04 (2 B, d, J_{BH} 150).

3-{2-[2-(2-Chloroethoxy)ethoxy]ethoxy}prop-2-yne **7**.—2-[2-(2-Chloroethoxy)ethoxy]ethanol **6** (6.74 g, 40 mmol) was added to sodium hydride (oil-free; 960 mg, 40 mmol) in dry THF (50 cm^3) at −20 °C. After 15 min at −78 °C, 3-bromopropyne (4.76 g, 40 mmol) was added to the mixture which was then boiled under reflux for 1 h. The mixture was evaporated and the residue was treated with water (50 cm^3) and extracted with dichloromethane. The extract was dried and evaporated and the residue was distilled to give the *title compound* **7** (5.83 g, 71%) as a colourless oil, b.p. 160 °C at 0.1 mmHg; ν_{max}/cm^{-1} 2120w; δ 2.44 (1 H, t, *J* 2.4, C≡CH), 3.6–3.8 (12 H, m, 5 × OCH_2 + $ClCH_2$) and 4.21 (2 H, d, *J* 2.4, $CH_2C{\equiv}C$); *m/z* (CI) 207.0788 (M + H) ($C_9H_{16}{}^{35}ClO_3$ requires 207.0788).

1-({2-[2-(2-Chloroethoxy)ethoxy]ethoxy}methyl)-1,2-di-carba-closo-dodecaborane(12) **8**.—Decaborane(14) ($B_{10}H_{14}$) (1.22 g, 10 mmol) was stirred with acetonitrile (20 cm^3) for 3 h before addition of the alkyne **7** (2.07 g, 10 mmol). The mixture was boiled under reflux for 3 d and then evaporated. Chromatography (pentane–dichloromethane 1:1) of the residue gave the *title compound* **8** (2.10 g, 65%) as a pale yellow oil; ν_{max}/cm^{-1} 2590; δ 1.3–3.1 (10 H, br m, $B_{10}H_{10}$), 3.6–3.8 (12 H, m, 5 × OCH_2 + $ClCH_2$), 3.96 (2 H, s, carborane-CH_2), and 4.07 (1 H, br s, carborane 2-H); *m/z* (CI) $^{10}B/^{11}B$ $^{35}Cl/^{37}Cl$ cluster centred at 326 (M); *m/z* (FAB, positive ion) 325.2587 (M + H) ($C_9H_{26}{}^{10}B_2{}^{11}B_8{}^{35}ClO_3$ requires 325.2574).

2-Hex-5-enyl-1-phenyl-1,2-dicarba-closo-dodecaborane(12) **10**.—Butyllithium (1.6 mol dm^{-3} in hexanes; 2.76 cm^3, 4.4 mmol) was added to 1-phenyl-1,2-dicarba-*closo*-dodecaborane(12) **4**[12] (720 mg, 3.3 mmol) in dry THF (10 cm^3) under nitrogen and the mixture was stirred for 30 min. 1-Bromohex-5-ene (720 mg, 4.4 mmol) was added to the mixture which was then stirred for 18 h. It was then washed with water and extracted with diethyl ether. The extract was evaporated and the residue chromatographed (pentane) to give the *title compound* **10** (800 mg, 81%) as a colourless oil; ν_{max}/cm^{-1} 3080, 2580 and 1640; δ 1.08 (2 H, quintet, *J* 7.3, $CH_2CH_2CH_2$), 1.2–3.3 (10 H, br m, $B_{10}H_{10}$), 1.34 (2 H, m, $CH_2CH_2CH_2$), 1.68 (2 H, m, $CH_2CH_2CH_2$), 1.80 (2 H, qt, *J* 7.5 and 1, $CH_2C{=}C$), 4.81 (2 H, m, $C{=}CH_2$), 5.55 (1 H, m, C=CH) and 7.25–7.57 (5 H, m, Ph-H_5); *m/z* (EI) 303.3010 (M) ($C_{14}H_{26}{}^{10}B^{11}B_9$ requires 303.3001), 302.3014 (M) ($C_{14}H_{26}{}^{10}B_2{}^{11}B_8$ requires 302.3034); *m/z* (CI) $^{10}B/^{11}B$ cluster centred at 605 (2M + H), $^{10}B/^{11}B$ cluster centred at 302 (M).

4,5-Dihydro-4-[4-(2-phenyl-1,2-dicarba-closo-dodecaboran-(12)-1-yl)butyl]-3-(3,4,5-trimethoxyphenyl)isoxazole **11**.—3,4,5-Trimethoxybenzaldehyde oxime **9**[19] (210 mg, 1 mmol) and the carborane **10** (300 mg, 1 mmol) in dichloromethane (20 cm^3) were stirred vigorously with aqueous sodium hypochlorite (8% available chlorine; 1.5 cm^3) for 16 h; further aqueous sodium hypochlorite (1.5 cm^3) was then added to the mixture. After 30 min, the mixture was diluted with water and extracted with dichloromethane. The extract was evaporated and the residue was chromatographed (pentane–dichloromethane, 3:1, then dichloromethane–ethyl acetate, 10:1, and then ethyl acetate). The starting carborane **10** (37%) and oxime **9** (29%) were isolated from the first and last portions of eluate, respectively. Evaporation of the solvent from the second portion of eluate gave the *title compound* **11** (300 mg, 59%) as a colourless oil; δ_H 1.0–3.2 (10 H, br m, $B_{10}H_{10}$), 1.26 (2 H, m, CH_2), 1.47 (4 H, m, 2 × CH_2), 1.79 (2 H, m, CH_2), 2.85 (1 H, dd, *J* 16.5 and 7.2) and 3.33 (1 H, dd, *J* 16.5 and 10.3) (isoxazole 4-H_2), 3.87 (9 H, s, 3 × OCH_3), 4.61 (1 H, m, isoxazole 5-H), 6.85 (2 H, s, Ar-H_2) and 7.35–7.64 (5 H, m, Ph-H_5); δ_C 24.7, 29.6, 34.7, 34.8, 40.0, 56.1, 56.1, 60.9, 80.7, 80.8, 82.1, 83.5, 103.8, 125.0, 128.9, 130.6, 131.0, 153.3 and 156.1; *m/z* (FAB, positive ion) $^{10}B/^{11}B$ cluster centred at 512 (M + H); *m/z* (FAB,

negative ion) 510.3657 (M − H) ($C_{24}H_{36}{}^{10}B_2{}^{11}B_8NO_4$ requires 510.3648).

3-Methoxy-2-prop-2-ynyloxybenzaldehyde Oxime **14**.—3-Methoxy-2-prop-2-ynyloxybenzaldehyde **12**[20] (1.16 g, 6.1 mmol) was stirred with sodium carbonate (640 mg, 6 mmol) and hydroxylamine hydrochloride (830 mg, 12 mmol) in ethanol (50 cm^3) for 16 h. The mixture was evaporated and the residue was dissolved in dichloromethane and the solution washed with water, dried and evaporated to give the *title compound* **14** (890 mg, 71%) as a white solid, m.p. 76–78 °C; ν_{max}/cm^{-1} 3270; δ 2.48 (1 H, t, *J* 2.4, C≡CH), 3.88 (3 H, s, OCH_3), 4.77 (2 H, d, *J* 2.4, $CH_2C{\equiv}C$), 6.95 (1 H, dd, *J* 8.2 and 1.5, Ar 4-H), 7.10 (1 H, t, *J* 8.1, Ar 5-H), 7.38 (1 H, dd, *J* 8.0 and 1.5, Ar 6-H), 8.21 (1 H, s, C*H*NOH) and 8.60 (1 H, s, OH). This material was used without further purification.

6-Methoxy-4H-[1]benzopyrano[4,3-c]isoxazole **16**.—The oxime **14** (150 mg, 0.75 mmol) in dichloromethane (10 cm^3) was stirred with aqueous sodium hypochlorite (8% available chlorine; 2 cm^3) for 48 h. The mixture was extracted with dichloromethane and the extract was washed with water and evaporated. Chromatography (dichloromethane–pentane, 1:1) of the residue gave the *title compound* **16** (20 mg, 13%) as a white solid, m.p. 126–128 °C; δ 3.92 (3 H, s, OCH_3), 5.33 (2 H, d, *J* 1.1, pyran-H_2), 6.99 (1 H, dd, *J* 8.1 and 1.8, ArH), 7.04 (1 H, t, *J* 8, ArH), 7.50 (1 H, dd, *J* 7.5 and 1.8, ArH) and 8.23 (1 H, t, *J* 1.1, isoxazole-H); *m/z* (EI) 203.1606 (M) ($C_{11}H_9NO_3$ requires 203.1582).

2-(3-Methoxy-2-prop-2-ynyloxyphenyl)-4,5-dihydro-1,3-dioxole **17**.—The aldehyde **12** (1.90 g, 10 mmol) was stirred at reflux with ethane-1,2-diol (680 mg, 11 mmol) and 4-methylbenzenesulfonic acid hydrate (10 mg) in toluene (25 cm^3) with azeotropic removal of water for 24 h. The mixture was evaporated and the residue chromatographed (dichloromethane–pentane, 1:1) to give the *title compound* **17** (2.11 g, 90%) as a white solid, m.p. 93–94 °C (Found: C, 66.55; H, 5.95. $C_{13}H_{14}O_4$ requires C, 66.65; H, 6.00%); δ 2.49 (1 H, br, C≡CH), 3.87 (3 H, s, OCH_3), 4.00–4.20 (4 H, m, CH_2CH_2), 4.77 (2 H, d, *J* 1.5, $CH_2C{\equiv}C$), 6.23 (1 H, s, dioxole 2-H) and 6.95–7.15 (3 H, m, Ar-H_3); *m/z* (EI) 234.0876 (M) ($C_{13}H_{14}O_4$ requires 234.0892).

2-(3-Methoxy-2-prop-2-ynyloxyphenyl)-4,5-dihydro-1,3-dithiole **19**.—The aldehyde **12** (950 mg, 5 mmol) and ethane-1,2-dithiol (940 mg, 10 mmol) in dry dichloromethane (10 cm^3) were treated dropwise with boron trifluoride–diethyl ether complex (710 mg, 5 mmol). The mixture was stirred for 24 h after which it was diluted with water (10 cm^3) and extracted with chloroform. The extract was evaporated and the residue chromatographed (pentane–dichloromethane, 4:1) to give the *title compound* **19** (700 mg, 53%) as a colourless oil; δ 2.49 (1 H, t, *J* 2.4, C≡CH), 3.30–3.54 (4 H, m, CH_2CH_2), 3.85 (3 H, s, OCH_3), 4.79 (2 H, d, *J* 2.4, $CH_2C{\equiv}C$), 6.21 (1 H, s, dithiole 2-H) and 6.81–7.35 (3 H, m, Ar-H_3).

2-[2-(1,2-Dicarba-closo-dodecaboran(12)-1-yl)methoxy-3-methoxyphenyl]-4,5-dihydro-1,3-dithiole **20**.—Decaborane(14) ($B_{10}H_{14}$) (120 mg, 1 mmol) was stirred in dry acetonitrile (10 cm^3) for 2 h after which the dithiole **19** (270 mg, 1 mmol) was added to it and the mixture boiled under reflux for 3 d. Evaporation of the mixture gave a residue which was chromatographed (pentane–dichloromethane, 10:1) and the resulting product recrystallised from hexane–benzene (CAUTION) to furnish the *title compound* **20** (200 mg, 52%) as white crystals, m.p. 132–133 °C (Found: C, 40.6; H, 6.35. $C_{13}H_{24}B_{10}O_2S_2$ requires C, 40.6; H, 6.25%); ν_{max}/cm^{-1} 2540; δ 1.0–3.2 (10 H, br m, $B_{10}H_{10}$), 3.41 (4 H, m, CH_2CH_2), 3.83 (3 H, s, OCH_3), 4.31 (1 H, br s, carborane 2-H), 4.44 (2 H, s, carborane-CH_2), 5.89 (1 H, s, dithiole 2-H) and 6.81–7.33 (3 H, m, Ar-H_3); *m/z* (FAB, positive ion) $^{10}B/^{11}B$ cluster centred at 385 (M + H).

4-Prop-2-ynyloxybenzaldehyde **22**.—4-Hydroxybenzaldehyde **21** (6.1 g, 50 mmol) was added to sodium ethoxide (50 mmol) in dry ethanol (100 cm^3), followed by 3-bromopropyne (6.55 g, 55 mmol). The mixture was boiled under reflux for 3 h and then evaporated. The residue was dissolved in dichloromethane and the solution washed with water, dried and evaporated to afford the *title compound* **22** (5.08 g, 64%) as a buff solid, m.p. 70–72 °C (lit.,[21] m.p. 79–80 °C); δ 2.58 (1 H, t, *J* 2.5, C≡CH), 4.79 (2 H, d, *J* 2.5, $CH_2C{\equiv}C$), 7.10 (2 H, d, *J* 8.8, Ar 3,5-H_2), 7.86 (2 H, d, *J* 8.8, A 2,6-H_2) and 9.91 (1 H, s, CHO).

2-(4-Prop-2-ynyloxyphenyl)-4,5-dihydro-1,3-dithiole **23**.—Boron trifluoride–diethyl ether complex (4.26 g, 30 mmol) was added to the aldehyde **22** (4.8 g, 30 mmol) and ethane-1,2-dithiol (5.64 g, 60 mmol) in dichloromethane (150 cm^2) at 0 °C and the mixture was stirred at ambient temperature for 16 h. It was then washed with water and evaporated. Chromatography (pentane–dichloromethane, 4:1) of the residue gave the *title compound* **23** (5.68 g, 80%) as a colourless oil; δ 2.52 (1 H, t, *J* 2.5, C≡CH), 3.33 (2 H, m) and 3.47 (2 H, m) (CH_2CH_2), 4.65 (2 H, d, *J* 2.5, $CH_2C{\equiv}C$), 5.63 (1 H, s, dithiole 2-H), 6.91 (2 H, d, *J* 8.8, Ar 3,5-H_2) and 7.46 (2 H, d, *J* 8.8, Ar 2,6-H_2). This material was used directly without further purification.

1-[4-(4,5-Dihydro-1,3-dithiol-2-yl)phenoxymethyl]-1,2-dicarba-closo-dodecaborane(12) **24**.—Decaborane(14) ($B_{10}H_{14}$) (2.44 g, 20 mmol) was stirred with acetonitrile (200 cm^3) for 3 h after which the dithiole **23** (4.72 g, 20 mmol) was added to the mixture. After being boiled under reflux for 3 d the mixture was evaporated and the residue chromatographed (pentane–dichloromethane, 4:1) to yield the *title compound* **24** (3.62 g, 51%) as a white solid, m.p. 123–125 °C (Found: C, 40.9; H, 6.2. $C_{12}H_{22}B_{10}OS_2$ requires C, 40.7; H, 6.3%); ν_{max}/cm^{-1} 2590; δ_H 1.5-3.0 (10 H, br m, $B_{10}H_{10}$), 3.34 (2 H, m) and 3.49 (2 H, m) (CH_2CH_2), 4.08 (1 H, br s, carborane 2-H), 4.39 (2 H, s, carborane-CH_2), 5.60 (1 H, s, dithiole 2-H), 6.77 (2 H, d, *J* 8.8, Ar 3,5-H_2) and 7.46 (2 H, d, *J* 8.8, Ar 2,6-H_2); δ_B (H-decoupled) −15.0 (4 B), −13.5 (2 B), −10.7 (2 B), −6.5 (1 B) and −4.5 (1 B); *m/z* (EI) $^{10}B/^{11}B$ cluster centred at 354 (M), $^{10}B^{11}B$ isotope cluster centred at 326 (M − C_2H_4).

1-(4-Formylphenoxymethyl)-1,2-dicarba-closo-dodecaborane(12) **25**.—The dithiole **24** (356 mg, 1 mmol) was stirred with mercury(II) perchlorate trihydrate (1.00 g, 2.2 mmol) in THF (8 cm^3) for 5 min. The suspension was then filtered and the filtrate evaporated to afford a residue which was dissolved in dichloromethane and the solution washed twice with aqueous sodium carbonate (10%), dried and evaporated to give the *title compound* **25** (250 mg, 90%) as white crystals, m.p. 148–151 °C (Found: C, 42.8; H, 6.5. $C_{10}H_{18}B_{10}O_2$ requires C, 40.7; H, 6.3%); ν_{max}/cm^{-1} 2580 and 1690; δ 1.3–3.4 (10 H, br m, $B_{10}H_{10}$), 4.07 (1 H, br s, carborane 2-H), 4.51 (2 H, s, carborane-CH_2), 6.97 (2 H, d, *J* 8.8, Ar 2,6-H_2), 7.87 (2 H, d, *J* 8.8, Ar 3,5-H_2) and 9.92 (1 H, s, CHO); *m/z* (EI) $^{10}B/^{11}B$ cluster centred at 278 (M).

1-[4-(Hydroxyiminomethyl)phenoxymethyl]-1,2-dicarba-closo-dodecaborane(12) **26**.—The aldehyde **25** (1.39 g, 5 mmol) was stirred with hydroxylamine hydrochloride (1.74 g, 25 mmol) and sodium carbonate (1.33 g, 12.5 mmol) in dry ethanol (50 cm^3) for 2 d after which the mixture was evaporated. The residue was dissolved in ethyl acetate and the solution washed with water and dried. Chromatography (dichloromethane) gave the title compound **26** (830 mg, 71%) as a white solid, m.p. 174–176 °C; ν_{max}/cm^{-1} 3300 and 2600; δ[$(CD_3)_2CO$] 1.3–3.4 (10 H,

br m, $B_{10}H_{10}$), 4.70 (2 H, s, carborane-CH_2), 5.00 (1 H, br s, carborane 2-H), 7.02 (2 H, d, *J* 9.1, Ar 2,6-H_2), 7.57 (2 H, d, *J* 9.1, Ar 3,5-H_2), 8.08 (1 H, s, C*H*NOH) and 10.19 (1 H, s, OH); *m/z* (EI) 295.2388 (M) ($C_{10}H_{19}{}^{11}B_{10}NO_2$ requires 295.2346), 294.2420 (M) ($C_{10}H_{19}{}^{10}B^{11}B_9NO_2$ requires 294.2381), 293.2449 (M) ($C_{10}H_{19}{}^{10}B_2{}^{11}B_8NO_2$ requires 293.2419) and 292.2454 (M) ($C_{10}H_{19}{}^{10}B_3{}^{11}B_7NO_2$ requires 292.2455).

1-(4-{3-[4-(1,2-*Dicarba*-closo-*dodecaboran*(12)-1-*ylmethoxy*)*phenyl*]-4,5-*dihydroisoxazol*-5-*yl*}*butyl*)-2-*nitroimidazole* **28** *and* 4-(1,2-*Dicarba*-closo-*dodecaboran*(12)-1-*ylmethoxy*)-*benzonitrile* N-*Oxide* **32**.—The oxime **26** (290 mg, 1 mmol) and 1-hex-5-enyl-2-nitroimidazole **27**[9] (200 mg, 1 mmol) in dichloromethane (8 cm^3) were stirred vigorously with aqueous sodium hypochlorite (8% available chlorine; 2 cm^3) for 18 h. Further aqueous sodium hypochlorite (6 cm^3) was added to the mixture which was then stirred for a further 1.5 h. It was then washed with water and evaporated and the residue chromatographed (pentane–dichloromethane, 1:1, then dichloromethane, then ethyl acetate) to give the *title compound* **32** (110 mg, 38%) (from the first fraction) as a white solid, m.p. 212–216 °C (decomp.); ν_{max}/cm^{-1} 2600 and 2310 cm^{-1}; δ 1.0–3.2 (10 H, br m, $B_{10}H_{10}$), 3.97 (1 H, br s, carborane 2-H), 4.38 (2 H, s, carborane-CH_2), 6.82 (2 H, d, *J* 9.0, Ar 3,5-H_2) and 7.40 (2 H, d, *J* 9.0, Ar 2,6-H_2); *m/z* (EI) 291.2262 (M) ($C_{10}H_{17}{}^{10}B_2{}^{11}B_8NO_2$ requires 291.2262), $^{10}B/^{11}B$ cluster centred on 291 (M), and $^{10}B/^{11}B$ isotope cluster centred on 275 (100%) (M − O). From the third fraction was obtained the *title compound* **28** (280 mg, 57%) as a gummy solid; ν_{max}/cm^{-1} 2600; δ 1.3–3.3 (10 H, br m, $B_{10}H_{10}$), 1.5–2.0 (6 H, m, imidazole-$CH_2CH_2CH_2CH_2$), 2.93 (1 H, dd, *J* 16.5 and 7.9) and 3.40 (1 H, dd, *J* 16.5 and 10.4) (isoxazole 4-H_2), 4.09 (1 H, br s, carborane 2-H), 4.45 (4 H, m, carborane-CH_2 + imidazole-CH_2), 4.73 (1 H, ddt, *J* 10, 8 and 7, isoxazole 5-H), 6.87 (2 H, d, *J* 9.0, Ar 3,5-H_2), 7.11 (1 H, s) and 7.15 (1 H, s) (imidazole 4,5-H_2) and 7.61 (2 H, s, *J* 9.0, Ar 2,6-H_2); *m/z* (FAB, positive ion) 487.3296 (M + H) ($C_{19}H_{31}{}^{10}B_2{}^{11}B_8N_4O_4$ requires 487.3348).

1-(2-{3-[4-(1,2-*Dicarba*-closo-*dodecaboran*(12)-1-*ylmethoxy*)*phenyl*]*isoxazol*-5-*yl*}*ethyl*)-2-*nitroimidazole* **29**.—The oxime **26** and the nitroimidazolealkyne **3** were treated with sodium hypochlorite, as for the synthesis of compound **28** above. Chromatography (pentane–dichloromethane, 1:1, then dichloromethane, then dichloromethane–diethyl ether, 2:1) gave the *N*-oxide **32** (38%) (from the first fraction) and then the *title compound* **29** (57%) (from the third fraction) as a white solid, m.p. 151–154 °C; ν_{max}/cm^{-1} 2590; δ 1.3–3.3 (10 H, br m, $B_{10}H_{10}$), 3.42 (2 H, t, *J* 6.7, imidazole-CH_2CH_2), 4.09 (1 H, br s, carborane 2-H), 4.46 (2 H, s, carborane-CH_2), 4.82 (2 H, t, *J* 6.7, imidazole-CH_2), 6.26 (1 H, s, isoxazole 4-H), 6.92 (2 H, d, *J* 8.8, Ar 3,5-H_2), 6.96 (1 H, s) and 7.09 (1 H, s) (imidazole 4,5-H_2) and 7.70 (2 H, s, *J* 9.0, Ar 2,6-H_2); *m/z* (EI) 458.2786 (M) ($C_{17}H_{24}{}^{11}B_{10}N_4O_4$ requires 458.2728), 457.2798 (M) ($C_{17}H_{24}{}^{10}B^{11}B_9N_4O_4$ requires 457.2764), 456.2827 (M) ($C_{17}H_{24}{}^{10}B_2{}^{11}B_8N_4O_4$ requires 456.2801), 455.2865 (M) ($C_{17}H_{24}{}^{10}B_3{}^{11}B_7N_4O_4$ requires 455.2837) and 454.2886 (M) ($C_{17}H_{24}{}^{10}B_4{}^{11}B_6N_4O_4$ requires 454.2873).

2-*Nitro*-1-{2-[2-(2-*prop*-2-*ynyloxyethoxy*)*ethoxy*]*ethyl*}*imidazole* **30**.—2-Nitroimidazole **2** (460 mg, 4 mmol) was stirred at 130 °C in DMF (10 cm^3) with potassium *tert*-butoxide (440 mg, 4 mmol) for 30 min. Potassium iodide (20 mg) and the alkyne **7** (840 mg, 4 mmol) were added to the mixture which was then stirred at 130 °C for 13 h. The mixture was evaporated and the residue was dissolved in dichloromethane and the solution washed with water, dried and evaporated. Chromatography (dichloromethane) of the residue gave the *title compound* **30** (490 mg, 43%) as a yellow oil; δ 2.45 (1 H, t, *J* 2.4, C≡CH), 3.60 (4 H, s) and 3.6–3.7 (4 H, m) (2 × OCH_2CH_2O), 3.86 (2 H, t, *J* 4.9, imidazole-CH_2CH_2), 4.20 (2 H, d, *J* 2.4, $CH_2C{\equiv}C$), 4.63 (2 H, t, *J* 4.9, imidazole-CH_2), and 7.14 (1 H, s) and 7.29 (1 H, s) (imidazole 4,5-H_2); *m/z* (CI) 284.1246 (M + H) ($C_{12}H_{18}N_3O_5$ requires 284.1246).

3-[4-(1,2-*Dicarba*-closo-*dodecaboran*(12)-1-*ylmethoxy*)-*phenyl*]-5-(2-{2-[2-(2-*nitroimidazol*-1-*yl*)*ethoxy*]*ethoxy*}*methyl*)*isoxazole* **31**.—The oxime **26** and the alkyne **30** were treated with sodium hypochlorite, as for the synthesis of compound **28** above. Chromatography (pentane–dichloromethane, 1:1, then dichloromethane–diethyl ether, 1:1) gave the *title compound* **31** (79%) as a pale yellow oil; ν_{max}/cm^{-1} 2600; δ [$(CD_3)_2SO$] 1.2–3.1 (10 H, br, $B_{10}H_{10}$), 3.45–3.65 (8 H, m, 2 × OCH_2CH_2O), 3.76 (2 H, t, *J* 5.1, imidazole-CH_2CH_2), 4.57 (2 H, t, *J* 5.1, imidazole-CH_2), 4.65 (2 H, s, carborane-CH_2), 5.37 (1 H, br s, carborane 2-H), 5.76 (1 H, s, isoxazole 4-H), 7.12 (2 H, d, *J* 9.0, Ar 2,6-H_2), 7.14 (1 H, d, *J* 1.1, imidazole 4-H), 7.62 (1 H, d, *J* 1.1, imidazole 5-H) and 7.84 (2 H, d, *J* 9.0, Ar 3,5-H_2); *m/z* (FAB, positive ion) 577.344 (M + H) (100%) ($C_{22}H_{35}{}^{11}B_{10}N_4O_7$ requires 577.344).

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Tumour-Targetted Boranes. Part 3. Synthesis of Carbamate-Linked Nitroimidazole-Carboranes Designed for Boron Neutron Capture Therapy of Cancer

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Tumour-targetted Boranes. Part 3.[1] Synthesis of Carbamate-linked Nitroimidazolyl Carboranes Designed for Boron Neutron Capture Therapy of Cancer

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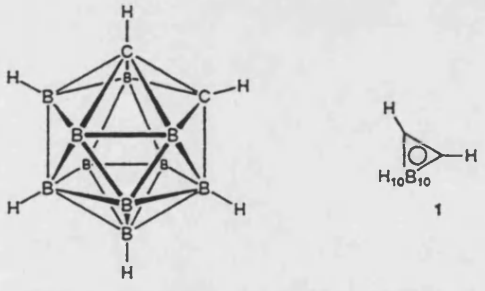
Carboranes targetted to specific tumour tissues are important for boron neutron capture therapy of cancer (BNCT). Carbamoylation of 2-[2-[2-(2-nitroimidazol-1-yl)ethoxy]ethoxy]ethanol **5** and 1-(chloromethyl)-2-(2-nitroimidazol-1-yl)ethanol **6** with carboran-1-yl isocyanate (generated *in situ* by a Curtius rearrangement of carborane-1-carbonyl azide) gave the corresponding carbamate-linked nitroimidazolylcarboranes **16** and **17**. A similar reaction of 4-carboranylphenyl isocyanate with **6** afforded the corresponding carbamate **24**.

Boron neutron capture therapy (BNCT) is of increasing interest for treatment of various cancers, mainly gliomas and melanomas.[2] When the ^{10}B isotope is irradiated with slow ('thermal') neutrons, an [n,α] reaction ensues, giving ^{7}Li and ^{4}He nuclei with kinetic energy (2.31 MeV). With this energy, the α-particle has a range of *ca.* 1 cell diameter in biological tissue and damage is limited to the cell containing the boron. Failures in early studies of BNCT were attributed[3] to inadequate concentrations of ^{10}B in the tumour tissue or to lack of selectivity of disposition of ^{10}B, leading to damage to normal tissue. Carboranes have been linked to nucleosides,[4] to amino acids[5] and to porphyrins[6] in attempts to target boron to tumours. 1-Substituted 2-nitroimidazoles are selectively retained in poorly vascularised hypoxic tumour tissue by reductive metabolism to electrophiles.[7] As part of a programme of synthesis and evaluation of nitroimidazoles in the treatment of cancer,[1,8] we sought compounds containing derivatives of 1,2-dicarba-*closo*-dodecaborane(12) ('carborane', **1**) linked to



1

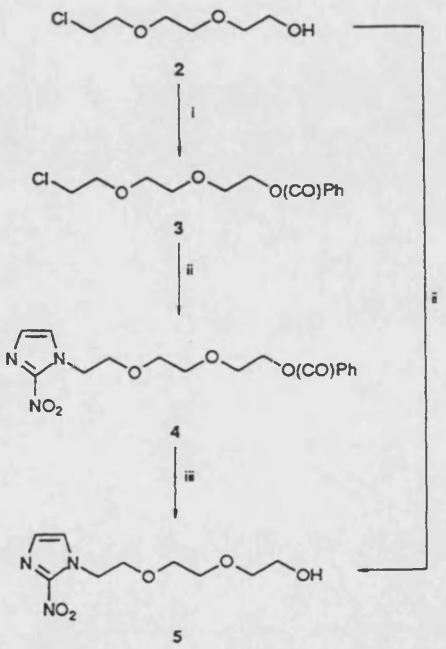
Representations of the structure of 1,2-dicarba-*closo*-dodecaborane(12) (carborane). Each boron atom has one hydrogen attached.

2-nitroimidazole as a strategy to cause selective retention of boron in hypoxic tumour tissue. Prior to our first preliminary communication,[9] no synthesis of a nitroimidazolylcarborane had been published, although reports of preparations of a nitroimidazolyl-*nido*-carborane and a nitroimidazolyl-*meta*-carborane with short links have been made in proceedings of conferences.[10]

Our previous study[1] showed that 'direct' methods of preparation of nitroimidazolylcarboranes, such as formation by treatment of a nitroimidazolylalkyne with decaborane(14) or alkylation of the 2-nitroimidazole anion with carboranyl electrophiles, were not feasible. The 1,3-dipolar cycloaddition of 4-(carboran-1-ylmethoxy)benzonitrile oxide with 1-(ω-alkynyl)-2-nitroimidazoles permitted coupling of the nitroimidazole and the carborane under mild conditions, although the product isoxazoles were insufficiently soluble in water to allow biological evaluation. We therefore sought an alternative mild linking strategy.

The addition of alcohols to isocyanates usually proceeds rapidly under mild conditions to give carbamates which are generally stable under physiological conditions. Addition of a nitroimidazole alcohol to a carborane carrying an isocyanate was therefore selected as a synthetic strategy.

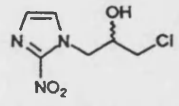
Two nitroimidazole alcohols, **5** and **6**, were prepared. To provide a suitable electrophile for reaction with the 2-nitroimidazole anion, the chloro alcohol **2** was protected as the benzoate ester **3** (Scheme 1). Treatment of this ester with the



Scheme 1 Synthesis of the nitroimidazole alcohol **5**: i, $PhCOCl$-Et_3N; ii, 2-nitroimidazole-KOBu^t-DMF; iii, NaOH

potassium salt of 2-nitroimidazole under the usual forcing conditions (DMF, 130 °C) gave the nitroimidazolyl ester **4** which was deprotected under basic conditions to give the required alcohol **5** in < 40% overall yield. Other protecting groups for the alcohol, including triphenylsilyl and tetrahydropyranyl, gave lower yields. Alkylation of the nitroimidazole anion with the unprotected chloro alcohol **2** was then

investigated and was found to give the nitroimidazole alcohol **5** in good yield. It was expected that the presence of the oxyethylene ether moiety in carbamoylated derivatives of this alcohol would contribute to the aqueous solubility of the target nitroimidazolylcarborane. The shorter chain substituted nitroimidazol-2-ylethanol **6** was prepared by reaction of 2-nitroimidazole with epichlorohydrin, by the method of Beaman *et al.*[11]

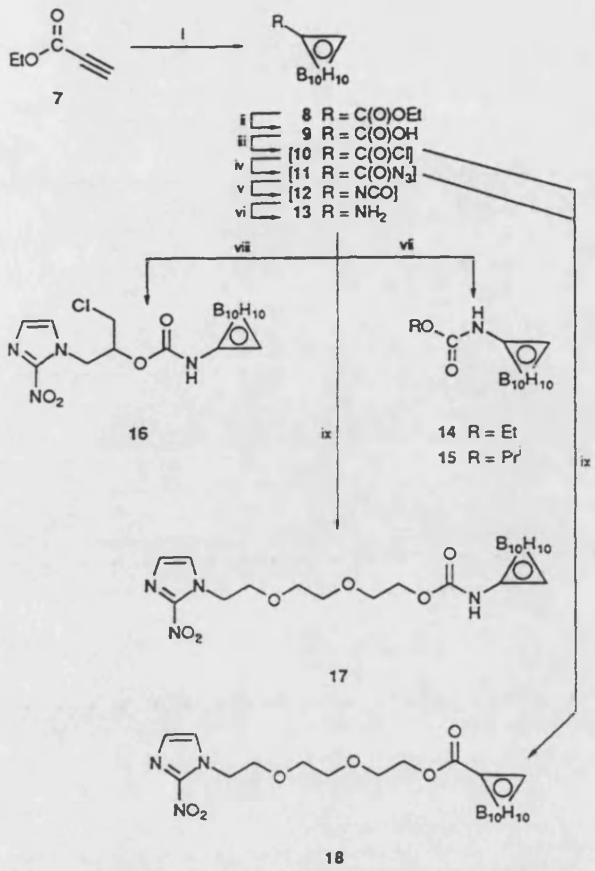


6

To provide a carborane with isocyanate directly attached to C-1, a strategy based on a Curtius rearrangement was employed. Following the standard method for the synthesis of carboranes from alkynes and decaborane(14) at elevated temperatures in the presence of a Lewis base,[12] the carborane ester **8** was formed from ethyl propynoate **7** and decaborane(14) in good yield. This ester was resistant to acid-catalysed hydrolysis but cleavage under mild basic conditions afforded the carboranecarboxylic acid **9** almost quantitatively. To establish a set of conditions for the Curtius rearrangement of the corresponding acid azide **11**, some preliminary experiments were undertaken. The acid chloride **10** was formed with thionyl chloride. Treatment with azidotrimethylsilane in boiling toluene, followed by hydrolysis of the isocyanate **12** with water in a one-pot process, gave the carboraneamine **13**[13] as a white solid which sublimed at 260–270 °C. In a similar run where ethanol replaced the added water, the major product was the expected ethyl carbamate **14**, formed from the isocyanate **12** generated *in situ*. A minor side-reaction was the trapping of unchanged acid chloride and/or acid azide to give the ethyl ester **8**. The 1-isopropyl carbamate **15** was formed analogously. These simple carbamates were found to be stable to both aqueous acid and aqueous base conditions, in experiments designed to confirm stability of the carborane carbamates under biological conditions. With conditions now established for generation and reaction of the carborane isocyanate with alcohols, the reactions with the nitroimidazole alcohols **5** and **6** were investigated. Treatment of the isocyanate **12** with the sterically hindered secondary alcohol **6** gave the desired carbamate-linked nitroimidazolylcarborane **16** in moderate yield. The yield was improved using the primary alcohol **5**, giving carbamate-linked nitroimidazolylcarborane **17**. A low yield of the corresponding ester **18** was also isolated, presumably arising from the acid chloride or the unrearranged acid azide.

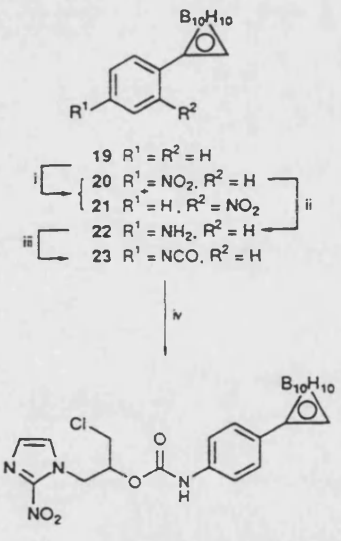
To provide a spacer between carborane and isocyanate units, the carboranylphenyl isocyanate **23** was synthesised. 1-Phenylcarborane **19** was nitrated using the mixed acid system but, in contrast to reports[14–16] that either a mixture of 3- and 4-nitro isomers or a mixture of 2-, 3- and 4-nitro compounds is produced, only the 2- and 4-nitrophenylcarboranes **21** and **20** were isolated in 2 and 85% yield, respectively. The isomers were characterised through their ^{1}H NMR spectra. The 4-nitro compound **20** was reduced with sodium borohydride and palladium, giving the 4-amine **22**. Treatment with phosgene under basic conditions afforded the phenyl isocyanate **23** which reacted smoothly with the secondary nitroimidazole alcohol **6** to furnish the required carbamate-linked nitroimidazolylcarborane **24**. In contrast, the reaction of the isocyanate **23** with the primary nitroimidazole alcohol **5** under the same conditions gave only inseparable mixtures.

The synthesis of the short series of carbamate-linked nitroimidazolylcarboranes **16**, **17** and **24** has demonstrated the feasibility of this type of coupling for the sensitive nitroimidazole and carborane moieties. The biological evaluation of these stable nitroimidazolylcarboranes will be reported elsewhere. The linkage of the nitroimidazolylalkyl carborane-



18

Scheme 2 Syntheses of the carbamate-linked nitroimidazolylcarboranes **16** and **17** and ester-linked nitroimidazolylcarborane **18**: i, $B_{10}H_{14}$–MeCN; ii, NaOH; iii, $SOCl_2$; iv, Me_3SiN_3; v, toluene and heat; vi, water, vii, EtOH or Pr^iOH; viii, **6**; ix, **5**



24

Scheme 3 Synthesis of the carbamate-linked nitroimidazolylcarborane **25**: i, HNO_3–H_2SO_4–CH_2Cl_2; ii, $NaBH_4$–Pd/C; iii, phosgene; iv, **6**

carboxylate **18** is relatively labile to hydrolysis under aqueous conditions and would not be capable of ensuring delivery of the boron to tumour tissue.

Experimental

Deuteriochloroform was the solvent for NMR spectroscopy with tetramethylsilane as chemical-shift standard, unless otherwise noted. Solutions in organic solvents were dried with anhydrous magnesium sulphate. Solvents were evaporated under reduced pressure. The chromatographic stationary phase was silica gel. M.p.s are uncorrected. DMF refers to dimethylformamide. *J* Values are recorded in Hz.

2-{2-[2-(2-*Nitroimidazol*-1-*yl*)*ethoxy*]*ethoxy*}*ethanol* **5**.—*Method A*. 2-Nitroimidazole (3.39 g, 30 mmol) was stirred with potassium *tert*-butoxide (3.36 g, 30 mmol) in DMF (50 cm^3) at 130 °C for 1 h after which the mixture was cooled to 80 °C. Sodium iodide (100 mg) and 2-[2-(2-chloroethoxy)ethoxy)]-ethanol **2** (6.74 g, 40 mmol) were added to the mixture which was then stirred at 130 °C for 2 h. The solvent was evaporated and the residue was dissolved in dichloromethane and the solution washed with water and dried. Chromatography (dichloromethane, then dichloromethane–ethyl acetate, 2:1, then ethyl acetate–methanol, 10:1) gave the *title compound* **5** (4.61 g, 63%) as a pale yellow oil; ν_{max}/cm^{-1} 3420br; δ 3.60 (4 H, s), 3.66 (2 H, m), 3.86 (2 H, t, *J* 5.0) and 4.22 (2 H, m) ($CH_2OCH_2CH_2OCH_2CH_2O$), 4.65 (3 H, m, imidazole-CH_2 + OH) and 7.18 (1 H, s) and 7.29 (1 H, s) (imidazole 4,5-H_2); *m/z* (CI) 246.1090 (M + H) ($C_9H_{16}N_3O_5$ requires 246.1090).

Method B. 2-[2-(2-Chloroethoxy)ethoxy]ethanol **2** (3.37 g, 20 mmol) was stirred with benzoyl chloride (2.81 g, 20 mmol) and triethylamine (2.02 g, 20 mmol) in dry dichloromethane (50 cm^3) for 24 h after which the mixture was washed with water, aqueous sodium hydroxide (2 mol dm^{-3}) and water and then dried. Chromatography (dichloromethane) gave 2-[2-(2-chloroethoxy)ethoxy]ethyl benzoate **3** (4.71 g, 86%) as a pale yellow oil; ν_{max}/cm^{-1} 1730; δ 3.61 (2 H, t, *J* 5.9), 3.71 (6 H, m) and 3.85 (2 H, m) ($ClCH_2CH_2OCH_2CH_2OCH_2$), 4.49 (2 H, m, CH_2O_2CPh), 7.45 (2 H, t, *J* 7.7, Ar 3,5-H_2), 7.57 (1 H, t, *J* 7.7, Ar 4-H) and 8.06 (2 H, d, *J* 7.7, Ar 2,6-H_2). 2-Nitroimidazole (1.13 g, 10 mmol) was heated at 130 °C with potassium *tert*-butoxide (1.12 g, 10 mmol) in DMF for 30 min after which the benzoate ester **3** (2.72 g, 10 mmol) was added to the mixture and the whole was stirred at 130 °C for 2 h. After this the solvent was evaporated and the residue was dissolved in dichloromethane and the solution washed with water and dried. Chromatography (dichloromethane, then dichloromethane–diethyl ether, 10:1) gave crude 2-{2-[2-(2-nitroimidazol-1-yl)ethoxy]ethoxy}ethyl benzoate **4**: δ 3.62 (4 H, m, $CH_2OCH_2CH_2OCH_2$), 3.82 (4 H, m, $CH_2OCH_2CH_2OCH_2$), 4.48 (2 H, t, *J* 5.0, CH_2O_2CPh), 4.57 (2 H, t, *J* 5.0) (imidazole-CH_2), 7.16 (1 H, s) and 7.21 (1 H, s) (imidazole 4,5-H_2), 7.45 (2 H, t, *J* 7.7, Ar 3,5-H_2), 7.57 (1 H, t, *J* 7.7, Ar 4-H) and 8.05 (2 H, d, *J* 7.7, Ar 2,6-H_2). This ester **4** (350 mg, 1 mmol) in ethanol (7.5 cm^3) was treated with aqueous sodium hydroxide (2 mol dm^{-3}, 2.5 cm^3, 5 mmol) at 50 °C for 1 h after which the ethanol was evaporated and the residue was diluted with water (10 cm^3) and extracted with dichloromethane. The extract was dried and the solvent was evaporated to give the *title compound* **5** (180 mg, 73%) with properties as above.

Ethyl 1,2-*Dicarba*-closo-*dodecaborane*(12)-1-*carboxylate* **8**.—Decaborane(14) (3.66 g, 30 mmol) was stirred with dry acetonitrile (50 cm^3) for 3 h after which ethyl propynoate **7** (2.94 g, 30 mmol) was added to the solution which was then stirred under reflux for 3 d. After this the mixture was evaporated and chromatography (pentane–dichloromethane, 2:1) of the residue gave the carborane ester **8** (3.26 g, 50%) as a white solid, m.p. 55–57 °C (lit.,[17] m.p. 62–62.5 °C); δ_H 1.32 (3 H, t, *J* 7.2, CH_3), 2.3 (10 H, br q, $J_{B,H}$ 150, $B_{10}H_{10}$), 4.08 (1 H, br s, carborane 2-H) and 4.29 (2 H, q, *J* 7.2, CH_2).

1,2-*Dicarba*-closo-*dodecaborane*(12)-1-*carboxylic Acid* **9**.—The ester **8** (2.16 g, 10 mmol) was stirred with sodium hydroxide (1.00 g, 25 mmol) in water (50 cm^3) and methanol (20 cm^3) for 3 d. The methanol was evaporated and the residue was acidified with hydrochloric acid (2 mol dm^{-3}) and extracted with dichloromethane. The extract was dried and the solvent was evaporated to give the carboranecarboxylic acid **9** (1.82 g, 97%) as a white solid, m.p. 148–150 °C (lit.,[18] m.p. 150 °C); δ[$(CD_3)_2SO$] 1.3–3.2 (10 H, br, $B_{10}H_{10}$), 5.20 (1 H, br s, carborane 2-H) and 9.5 (1 H, CO_2H).

Ethyl N-(1,2-*Dicarba*-closo-*dodecaboran*(12)-1-*yl*)*carbamate* **14**.—The carboranecarboxylic acid **9** (380 mg, 2 mmol) was boiled under reflux with thionyl chloride (10 cm^3) and DMF (0.03 cm^3) for 4 h. The excess of reagents was evaporated and the residue, dissolved in toluene (10 cm^3), was treated with azidotrimethylsilane (250 mg, 2.2 mmol) at reflux for 6 h. The mixture was cooled and ethanol (2 cm^3) was added to it; it was then boiled under reflux for 1 h. The solvents were evaporated and the residue was subjected to chromatography (pentane–dichloromethane, 2:1, then pentane–dichloromethane, 1:3). From the first fraction was obtained ethyl 1,2-dicarba-*closo*-dodecaborane(12)-1-carboxylate **8** (70 mg, 16%). From the second fraction was isolated the *title compound* **14** (270 mg, 58%) as a colourless solid. A sample of this material was recrystallised from light petroleum (b.p. 60–80 °C): m.p. 102–104 °C (Found: C, 26.0; H, 7.5; N, 6.3. Calc. for $C_5H_{17}B_{10}NO_2$: C, 25.95; H, 7.36; N, 6.06); ν_{max}/cm^{-1} 3290, 2600 and 1710; δ_H 1.26 (3 H, t, *J* 7.1, CH_3), 2.2 (10 H, br q, $J_{B,H}$ 150, $B_{10}H_{10}$), 4.13 (2 H, q, *J* 7.1, CH_2), 4.59 (1 H, br s, carborane 2-H) and 5.90 (1 H, br s, NH); *m/z* (EI) $^{10}B/^{11}B$ isotope cluster centred at 230 (M − H).

Isopropyl N-(1,2-*Dicarba*-closo-*dodecaboran*(12)-1-*yl*)*carbamate* **15**.—A solution of the isocyanate **12** in toluene was treated with propan-2-ol as for the synthesis of the ethyl carbamate **14**, except that chromatography was omitted, to give the title compound **15** (290 mg, 59%) as a white solid, m.p. 148–150 °C (Found: C, 29.6; H, 7.9; N, 5.9. Calc. for $C_6H_{19}B_{10}NO_2$ C, 29.39; H, 7.76; N, 5.71); ν_{max}/cm^{-1} 3330, 2605 and 1710; δ[$(CD_3)_2SO$] 1.18 [6 H, d, *J* 6.2, $(CH_3)_2$], 1.0–3.0 (10 H, br, $B_{10}H_{10}$), 4.78 (1 H, septet, *J* 6.2, $CHMe_2$), 5.35 (1 H, br s, carborane 2-H) and 9.66 (1 H, s, NH); *m/z* (EI) $^{10}B/^{11}B$ isotope cluster centred at 244 (M − H).

1-(*Chloromethyl*)-2-(2-*nitroimidazol*-1-*yl*)*ethyl* N-(1,2-*Dicarba*-closo-*dodecaboran*(12)-1-*yl*)*carbamate* **16**.—The carboranecarboxylic acid **9** (380 mg, 2 mmol) was boiled under reflux for 4 h with thionyl chloride (10 cm^3) and dimethylformamide (0.03 cm^3). The excess of reagents was evaporated and the residue, in toluene (10 cm^3), was treated with azidotrimethylsilane (250 mg, 2.2 mmol). The mixture was boiled under reflux for 18 h and then cooled to ambient temperature. 1-(Chloromethyl)-2-(2-nitroimidazol-1-yl)ethanol **6**[11] (410 mg, 2 mmol) was added to the mixture which was then boiled under reflux for 1 h. After evaporation the residue, in chloroform, was cooled to 0 °C. The solid was collected to give the title compound **16** (290 mg, 38%) as a white solid, m.p. 190–193 °C (Found: C, 27.7; H, 4.8. Calc. for $C_9H_{20}B_{10}ClN_4O_4$ C, 27.66; H, 4.87); ν_{max}/cm^{-1} 3160, 2600 and 1740; δ[$(CD_3)_2SO$] 1.5–2.8 (10 H, br, $B_{10}H_{10}$), 3.80 (1 H, dd, *J* 12.2 and 6.4) and 3.94 (1 H, dd, *J* 12.2 and 4.0) (CH_2Cl), 4.46 (1 H, dd, *J* 14.3 and 9.2) and 4.83 (1 H, dd, *J* 14.3 and 3.0) (imidazole-CH_2), 5.12 (1 H, br s, carborane 2-H), 5.30

(1 H, m, CHOH), 7.15 (1 H, s, imidazole 4-H), 7.43 (1 H, s, imidazole 5-H) and 9.98 (1 H, s, NH); *m/z* (EI) 344 (M − HCl) and 184 (carborane-N=C=O); *m/z* (FAB +ve ion) $^{10}B/^{11}B$ isotope cluster centred at 391 (M + H), 393.2087 (M + H) ($C_9H_{20}{}^{11}B_{10}{}^{35}ClN_4O_4$ requires 393.2104).

2-{2-[2-(2-Nitroimidazole-1-yl)ethoxy]ethoxy}ethyl N-(1,2-*Dicarba*-closo-*dodecaboran*(12)-1-*yl*)*carbamate* **17** *and* 2-{2-[2-(2-*Nitroimidazol*-1-*yl*)*ethoxy*]*ethoxy*}*ethyl* 1,2-*Dicarba*-closo-*dodecaborane*(12)-1-*carboxylate* **18**.—The carboranecarboxylic acid **9** (380 mg, 2 mmol) was boiled under reflux for 4 h with dimethylformamide (0.03 cm^3) and thionyl chloride (10 cm^3). The excess of reagent was evaporated and the residue was boiled under reflux with azidotrimethylsilane (250 mg, 2.2 mmol) in toluene (10 cm^3) for 16 h. The alcohol **5** (490 mg, 2 mmol) was added to the mixture and heating was continued for a further 2 h. The residue on evaporation was subjected to chromatography (pentane–dichloromethane, 2:1, then dichloromethane, then dichloromethane–diethyl ether). From the dichloromethane fraction was obtained the *title compound* **18** (141 mg, 17%) as a pale yellow gum; ν_{max}/cm^{-1} 2600, 1750; δ 2.3 (10 H, br q, $J_{B,H}$ 150 Hz, $B_{10}H_{10}$), 3.57 (4 H, s) and 3.65 (2 H, m) and 3.84 (2 H, t, *J* 5.0) ($CH_2OCH_2CH_2OCH_2$), 4.13 (1 H, br s, carborane 2 H), 4.37 (2 H, m, CO_2CH_2), 4.64 (2 H, t, *J* 5.0 Hz, imidazole-CH_2) and 7.14 (1 H, s) and 7.21 (1 H, s) (imidazole 4,5-H_2); *m/z* (FAB + ve ion) 416.2830 (M + H) ($C_{12}H_{26}{}^{10}B_2{}^{11}B_8N_3O_6$ requires 416.2824). From the dichloromethane–diethyl ether fraction was obtained the *title compound* **17** (47%) as a pale yellow gum; ν_{max}/cm^{-1} 3250, 2600 and 1755 cm^{-1}; δ 2.2 (10 H, br q, $J_{B,H}$ 150, $B_{10}H_{10}$), 3.4–3.6 (6 H, m) and 3.81 (2 H, t, *J* 5.0) and 4.10 (2 H, m) ($CO_2CH_2CH_2OCH_2CH_2OCH_2$), 4.62 (1 H, br s, carborane 2-H), 4.66 (2 H, t, *J* 5.0, imidazole-CH_2), 7.12 (1 H, d, *J* 0.8) and 7.16 (1 H, d, *J* 0.8) (imidazole 4,5-H_2) and 8.10 (1 H, br s, NH); *m/z* (FAB + ve ion) 431.2941 (M + H) ($C_{12}H_{27}{}^{10}B_2{}^{11}B_8N_4O_6$ requires 431.2933).

1-(4-*Nitrophenyl*)-1,2-*dicarba*-closo-*dodecaborane*(12) **20** *and* 1-(2-*Nitrophenyl*)-1,2-*dicarba*-closo-*dodecaborane*(12) **21**.—1-Phenyl-1,2-dicarba-*closo*-dodecaborane(12) **19**[12] in dichloromethane (50 cm^3) was stirred vigorously with nitric acid (66%; 6.0 cm^3) and concentrated sulphuric acid (34 cm^3) for 24 h. The organic phase was washed with water and with saturated aqueous sodium hydrogen carbonate and then dried. The residue on evaporation was subjected to chromatography (pentane–dichloromethane, 7:1, then pentane–dichloromethane 1:1). From the first fraction was obtained the title compound **21** (40 mg, 2%) as a white solid, m.p. 159–161 °C (lit.,[16] m.p. 161–161.5 °C); δ 2.3 (10 H, br q, $J_{B,H}$ 150 Hz, $B_{10}H_{10}$), 4.31 (1 H, br s, carborane 2-H), 7.39 (1 H, m, Ar 6-H), 7.55 (2 H, m, Ar 4,5-H_2) and 7.90 (1 H, m, Ar 3 H). From the second fraction was isolated the title compound **20** (2.25 g, 85%) as a white solid, m.p. 165–166 °C (lit.,[19] m.p. 167–168 °C); δ 2.3 (10 H, br q, $J_{B,H}$ 150, $B_{10}H_{10}$), 4.07 (1 H, br s, carborane 2-H), 7.68 (2 H, d, *J* 8.8, Ar 2,6-H_2) and 8.21 (2 H, d, *J* 8.8, Ar 3,5-H_2).

4-(1,2-*Dicarba*-closo-*dodecaboran*(12)-1-*yl*)*aniline* **22**.—Sodium borohydride (3.04 g, 8 mmol) in water (5 cm^3) was added to the 4-nitro compound **20** (2.12 g, 8 mmol) and 10% palladium-on-charcoal (210 mg) in methanol (100 cm^3) and the mixture was stirred for 16 h under nitrogen. The suspension was filtered through Celite®. The residue on evaporation in dichloromethane, was washed with water and dried. The solvent was evaporated to give the amine **22** (1.82 g, 77%) as a white solid: m.p. 101–103 °C (lit.,[15] m.p. 103–105 °C); δ 2.2 (10 H, br q, $J_{B,H}$ 150, $B_{10}H_{10}$), 4.20 (1 H, br s, carborane 2-H), 6.64 (2 H, d, *J* 8.6, Ar 2,6-H_2), 7.36 (2 H, d, *J* 8.6, Ar 3,5-H_2) and 8.5 (2 H, br, NH_2).

4-(1,2-*Dicarba*-closo-*dodecaboran*(12)-1-*yl*)*phenyl Isocyanate* **23**.—The amine **22** (350 mg, 1.5 mmol) in toluene (5 cm^3) was treated with phosgene in toluene (20%; 0.9 cm^3, 1.7 mmol) and triethylamine (303 mg, 3 mmol) under reflux for 2 h. The mixture was cooled and filtered. The solvent was evaporated to afford the isocyanate **23** (400 mg, quant.) as a pale buff solid, m.p. 131–134 °C (reported by Sergeev *et al.*[20] but no m.p. was given); ν_{max}/cm^{-1} 2580 and 2280; δ_H 2.3 (10 H, br q, $J_{B,H}$ 150, $B_{10}H_{10}$), 3.92 (1 H, br s, carborane 2-H), 7.05 (2 H, d, *J* 9.0, Ar 2,6-H_2) and 7.44 (2 H, d, *J* 9.0, Ar 3,5-H_2); *m/z* (EI) $^{10}B/^{11}B$ isotope cluster centred at 261 (M). This material was used directly without further characterisation.

1-(*Chloromethyl*)-2-(2-*nitroimidazol*-1-*yl*)*ethyl* N-(4-(1,2-*Dicarba*-closo-*dodecaboran*(12)-1-*yl*)*phenyl*)*carbamate* **24**.—The isocyanate **23** (130 mg, 500 μmol) was boiled under reflux with 1-(chloromethyl)-2-(2-nitroimidazol-1-yl)ethanol **6**[11] (100 mg, 500 μmol) in toluene (10 cm^3) for 24 h. The solvent was evaporated and the residue was recrystallised from chloroform to give the title compound **24** (190 mg, 81%) as a white solid: m.p. 177–180 °C (decomp.); ν_{max}/cm^{-1} 3270, 2600 and 1740; δ_H[$(CD_3)_2SO$] 1.3–3.0 (10 H, br, $B_{10}H_{10}$), 3.89 (1 H, dd, *J* 12.1 and 5.9) and 4.04 (1 H, dd, *J* 12.1 and 3.9 Hz) (CH_2Cl), 4.63 (1 H, dd, *J* 14.3 and 8.8) and 4.88 (1 H, dd, *J* 14.3 and 2.5 Hz) (imidazole-CH_2), 5.44 (1 H, m, CHO_2C), 5.67 (1 H, br s, carborane 2-H), 7.14 (1 H, s, imidazole 4-H), 7.33 (2 H, d, *J* 8.8) and 7.47 (2 H, d, *J* 8.8) (Ar-H_4), 7.57 (1 H, s, imidazole 5-H) and 10.03 (1 H, br s, NH); *m/z* (FAB +ve ion) 468.2355 (*M* + H) ($C_{15}H_{24}{}^{11}B_{10}{}^{35}ClN_4O_4$ requires 468.2338).

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PUBLICATION 62

Labelled Compounds of Interest as Antitumour Agents. Part 4. Deuteration and Tritiation of a Nitroimidazole-Carborane Designed for BNCT

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Labelled Compounds of Interest as Antitumour Agents. Part 4[1]. Deuteration and Tritiation of a Nitroimidazole-Carborane Designed for BNCT.

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Summary

Quenching the anion generated from a 2-(ω-carboranylalkyl)dithiane with ^2H_2O at -78°C and at 0°C introduced deuterium exclusively at C-2 of the carborane. Extension of this model reaction to a bioreductively-targetted carborane allowed the synthesis of 2-[^{2}H]- and 2-[^{3}H]-isotopomers of a nitroimidazole-carborane which is of interest in boron neutron capture therapy (BNCT) of cancer.

Keywords: Nitroimidazole, carborane, boron neutron capture therapy, hypoxia, deuterium oxide, tritiated water.

Introduction

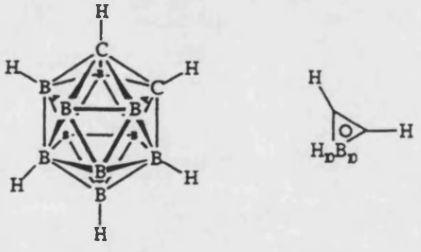

Figure 1. Representations of the structure of carborane. Each B atom has one H atom attached.

Boron neutron capture therapy (BNCT) is of increasing interest in the treatment of various cancers[2-4]. Failures in the early clinical studies were attributed[5-7] to inadequate concentration of the ^{10}B isotope in the tumour tissue or to lack of selectivity of disposition of ^{10}B, leading to damage to normal tissue. To exploit the selective retention[8-12] of 1-substituted 2-nitroimidazoles in hypoxic tumour tissue, we have synthesised[1,13,14] a series of carboranes linked to nitroimidazole. Figure 1 shows the icosahedral structure of carborane. We required radiolabelled material for studies on the biodistribution and selective localisation of the lead compound, the isoxazole-linked nitroimidazole-carborane **3** (Scheme 2). Owing to the complexity of the structure of **3** and the possible consequent complexity of its metabolism, we chose to radiolabel the carborane moiety, since investigation of the biodistribution of the boron was the objective of our study.

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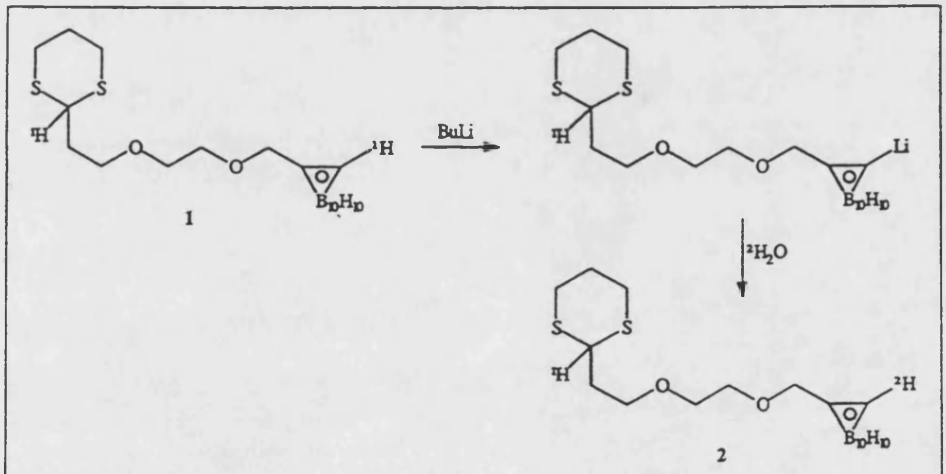
Mizusawa *et al.*[15] have reported tritiation of 1-phenylcarborane by formation of the anion at position 2 of the carborane with butyl lithium at elevated temperature and quenching with tritiated water.

Results and Discussion

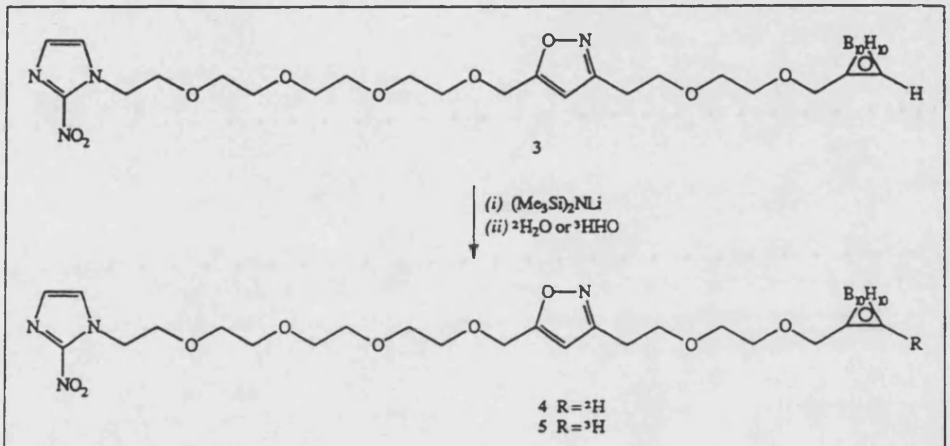
Before embarking on a deprotonation / quenching sequence on the complex molecule 3, a model study was performed. The dithiane-carborane 1 was synthesised in three steps (alkylation of dithiane anion with 1,2-bis(2-chloroethoxy)ethane, substitution of the chlorine by propyn-3-ol and treatment of the dithiane-alkyne with decaborane(14)) as previously described[16]. Treatment of 1 with one equivalent of butyl lithium at -78°C and quench with deuterium oxide gave exclusively an isotopomer containing only one deuterium (Scheme 1). Deprotonation and reaction at 0°C gave the same material. The vigorous conditions of Mizusawa *et al.*[15] were found to be unnecessary for deprotonation. Since both dithianes[16] and carboranes[15] are known to be deprotonated by butyl lithium, it was important to locate the deuterium by NMR spectroscopy. In the ^1H NMR spectrum, the dithiane 2-H was evident as a triplet at δ 4.18 but the broad singlet normally observed at δ 4.1 for the carborane 2-H in deuteriochloroform solvent was absent. The ^{13}C spectrum confirmed the location of the deuterium. The dithiane 2-C appeared as a singlet at δ 43.77 in the broadband ^1H-decoupled spectrum, whereas the carborane 2-C gave a 1:1:1 triplet (J = 29 Hz) centred at δ 57.36.



Scheme 1. Deuteration of the dithiane-carborane 1 and demonstration that the carborane 2-H is more acidic than the dithiane 2-H.

Since 2-nitroimidazole has electrophilic reactivity, lithium hexamethyldisilazide was used as a hindered non-nucleophilic base for the deprotonation of the nitroimidazole-carborane 3. Deprotonation at low temperature, followed by quench with deuterium oxide gave a monodeuterio isotopomer. NMR spectroscopy was again used to locate the deuterium, as deprotonation of the isoxazole at the 4-position is conceivable. The ^1H spectrum showed the absence of the carborane 2-H signal at δ 4.1 and the presence of the isoxazole 4-H signal at δ 6.18. The ^{13}C spectrum showed the carborane C-2 signal as the expected 1:1:1 triplet (J = 30 Hz) centred at δ 57.54.

A similar deprotonation of 3, followed by quench with tritiated water (specific activity 1.8 mCi mmol^{-1}) gave the tritiated isotopomer 5, although in disappointing chemical yield. Since the deprotonation is shown by the deuterium incorporation model experiment to be quantitative, the specific activity of 5 (248 μCi mmol^{-1}) indicates a significant kinetic tritium isotope effect on the reaction of the carborane anion with the water.



Scheme 2. Deuteration and tritiation of the nitroimidazole-carborane 3.

This synthesis of the radiolabelled nitroimidazole-carborane 5 provides material for quantitative biodistribution studies to confirm the preliminary biodistribution studies by ^{11}B magnetic resonance spectroscopy *in vivo* of tumour-bearing mice which had received the carborane 3. These results will be reported elsewhere.

Experimental Section

Tritiated water (1.8 mCi mmol^{-1}, 67 MBq mmol^{-1}) was obtained from ICN; deuterium oxide (99 atom %) was obtained from Aldrich Chemical Co. Ltd. Solutions in dichloromethane were dried with anhydrous magnesium sulphate. Solvents were evaporated under reduced pressure. THF refers to tetrahydrofuran; brine refers to saturated aqueous sodium chloride. NMR spectra were recorded of solutions in $CDCl_3$ with $SiMe_4$ as internal standard using Jeol GX270 and EX400 instruments. IR spectra were recorded on samples as liquid films.

2-[^{2}H]-1-(2-(2-(Dihydro-1,3-dithian-2-yl)ethoxy)ethoxymethyl)-1,2-dicarba*closo*dodecaborane(12) (2). Butyl lithium (2 M in hexanes, 0.25 mL, 0.5 mmol) was added to 1-(2-(2-(dihydro-1,3-dithian-2-yl)ethoxymethyl)ethoxymethyl)-1,2-dicarba*closo*dodecaborane(12) (**1**)[16] (180 mg, 0.5 mmol) in dry THF (5 mL) at -78°C under nitrogen and the mixture was stirred at -78°C for 45 min. Deuterium oxide (0.25 mL) was added and the mixture was warmed to ambient temperature during 30 min. Brine (5 mL) was added and the mixture was extracted with dichloromethane. The

extract was dried and the solvent was evaporated to give 2-[^{2}H]-1-(2-(2-(dihydro-1,3-dithian-2-yl)ethoxy)ethoxymethyl)-1,2-dicarba*closo*dodecaborane(12) (2) (180 mg, quant.) as a pale yellow gum: IR ν 2600 (B-H), 2260 (C-D) cm^{-1}; NMR δ_H 1.87 (1 H, m, dithiane 5_{ax}-H), 2.00 (2 H, q, J = 7.0 Hz, dithiane-CH_2), 2.14 (1 H, m, dithiane 5_{eq}-H) 2.3 (10 H, br q J_{B-H} = 160 Hz, $B_{10}H_{10}$), 2.85 (4 H, m, dithiane 4,6-H_4), 3.60 (6 H, m, dithiane-$CH_2CH_2OCH_2CH_2$), 3.96 (2 H, s, carborane-CH_2), 4.18 (1 H, t, J = 7.0 Hz, dithiane 2-H; NMR δ_C 25.74 (CH_2), 30.00 (CH_2), 35.31 (CH_2), 43.77 (dithiane 2-C), 57.36 (t, J_{C-D} = 29 Hz, carborane 2-CD), 67.24 (CH_2), 69.90 (CH_2), 71.18 (CH_2), 72.01 (CH_2), 72.72 (carborane 1-C); mass spectrum (CI) *m/z* ^{10}B/^{11}B isotope cluster centred at 366 (*M* + H).

2-[^{2}H]-1-(2-(2-(5-(2-(2-(2-(2-Nitroimidazol-1-yl)ethoxy)ethoxy)ethoxy)ethoxymethyl)-isoxazol-3-yl)ethyl)ethoxymethyl)-1,2-dicarba*closo*dodecaborane(12) (4). Butyl lithium (2 M in hexanes, 0.05 mL, 0.1 mmol) was added to hexamethyldisilazane (16 mg, 0.1 mmol) in dry THF at -78°C under nitrogen and the mixture was stirred at this temperature for 30 min. The unlabelled nitroimidazole-carborane 3[16] (60 mg, 0.1 mmol) in dry THF (2 mL) was added and stirring continued at -78°C for 30 min. Deuterium oxide (0.2 mL) was added and the mixture was warmed to ambient temperature during 30 min. Brine (5 mL) was added and the mixture was extracted with dichloromethane. The extract was dried and the solvent was evaporated to give 2-[^{2}H]-1-(2-(2-(5-(2-(2-(2-(2-nitroimidazol-1-yl)ethoxy)ethoxy)ethoxy)ethoxymethyl)isoxazol-3-yl)ethyl)ethoxymethyl)-1,2-dicarba*closo*dodecaborane(12) (4) (50 mg, 83%) as a pale yellow gum: IR ν 2590 (B-H), 2270 (C-D) cm^{-1}; NMR δ_H 2.3 (10 H, br q, J_{B-H} = 160 Hz, $B_{10}H_{10}$), 2.92 (2 H, t, J = 6.4 Hz, isoxazole-3-CH_2), 3.60-3.75 (18 H, m, carborane-$CH_2OCH_2CH_2OCH_2$ + $OCH_2CH_2OCH_2CH_2OCH_2CH_2O$), 3.85 (2 H, t, J = 5.0 Hz, imidazole-CH_2CH_2), 3.92 (2 H, s, carborane-CH_2), 4.62 (4 H, m, imidazole-CH_2 + isoxazole-5-CH_2O) 6.18 (1 H, s, isoxazole 4-H), 7.11 (1 H, d, J = 0.8 Hz) and 7.28 (1 H, d, J = 0.8 Hz) (imidazole 4,5-H_2); δ_C 26.75 (CH_2), 29.62 (CH_2), 49.82 (CH_2), 57.54 (t, J_{C-D} = 30 Hz, carborane 2-CD), 61.66 (CH_2), 63.98 (CH_2), 69.95 (CH_2), 69.32 (CH_2), 70.05 (CH_2), 70.31 (CH_2), 70.40 (CH_2), 70.47 (CH_2), 70.58 (CH_2), 71.26 (CH_2), 72.26 (CH_2), 72.52 (carborane 1-C), 103.08 (CH), 127.27 (CH), 127.93 (CH), 161.22 (C_q), 169.03 (C_q); mass spectrum (FAB) *m/z* ^{10}B/^{11}B isotope cluster centred at 616 (*M* + H).

2-[^{3}H]-1-(2-(2-(5-(2-(2-(2-(2-Nitroimidazol-1-yl)ethoxy)ethoxy)ethoxy)ethoxymethyl)-isoxazol-3-yl)ethyl)ethoxymethyl)-1,2-dicarba*closo*dodecaborane(12) (5). The unlabelled nitroimidazole-carborane 3[16] (60 mg, 0.1 mmol) was deprotonated with lithium hexamethyldisilazide, as for the synthesis of the deuterium isotopomer 4, Tritiated water (0.025 mL, 25 mCi, 925 MBq) was added and the mixture warmed to ambient temperature during 1 h. Brine (10 mL) was added and the mixture was extracted with dichloromethane. The extract was dried and the solvent was evaporated. Column chromatography (silica gel, ethyl acetate / methanol 10:1) gave 2-[^{3}H]-1-(2-(2-(5-(2-(2-(2-(2-nitroimidazol-1-yl)ethoxy)ethoxy)ethoxy)ethoxymethyl)isoxazol-3-yl)ethyl)ethoxymethyl)-1,2-dicarba*closo*dodecaborane(12) (5) (7.8 mg, 13% chemical yield, 3.15 μCi, 248 μCi mmol^{-1}, 9.18 MBq mmol^{-1}) as a colourless gum, identical by t.l.c. with 3 and 4.

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PUBLICATION 63

Tumor-Targeted Boranes. 4. Synthesis of Nitroimidazole-Carboranes with Polyether-Isoxazole links

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Journal of Organic Chemistry, **1994**, *59*, 7008-7013.

Tumor-Targeted Boranes. 4.[1] Synthesis of Nitroimidazole-Carboranes with Polyether-Isoxazole Links

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Carboranes targeted to specific tumor tissues are important for boron neutron capture therapy (BNCT) of cancer. Previous attempts to use isoxazolylphenyl-linked nitroimidazole-carboranes for targeting to hypoxic tumors were hampered by the low polarity and very low aqueous solubility of the compounds. Syntheses of polyether-linked nitroimidazole-isoxazole-carborane **17**, **25**, and **26** *via* 1,3-dipolar cycloaddition of appropriate nitroimidazole-alkynes with nitrile oxides derived from aliphatic aldehyde oximes linked by a varying number of water-solubilizing ether units to carborane have been developed. The stabilities of the nitrile oxides were much less than that of the corresponding 4-(carboranylmethoxy)phenyl nitrile oxide and depended on their structure. The yields of isoxazoles varied accordingly and cycloaddition failed when eight ether units were included in the chain. Compounds **17**, **25**, and **26**, with four, five, and six ether units, respectively, had increasingly convenient physical properties to permit biological evaluation.

Introduction

Boron neutron capture therapy (BNCT) is of increasing interest for the treatment of cancer.[2-5] Naturally occurring boron comprises two stable isotopes. ^{11}B, present at 80% abundance, is a convenient nucleus for NMR studies in vitro (sensitivity 0.17 × ^1H, $I = 3/2$). However, when ^{10}B is irradiated with low-energy ("thermal") neutrons, the ^{10}B nucleus captures a neutron and an [n,α] reaction ensues, giving ^7Li and ^4He nuclei, with kinetic energy (2.31 MeV). With this kinetic energy, the α-particles have ranges comparable with one cell diameter in biological tissue. Thus damage should be limited to cells containing the boron. Major endogenous nuclei (^1H, ^{12}C, ^{14}N, ^{16}O) have relatively very small nuclear cross-sections for neutron capture. Failures in early clinical studies of BNCT have been attributed[6-8] to inadequate concentrations of ^{10}B in the tumor tissue at the time of irradiation with neutrons or to lack of selectivity of biodistribution of ^{10}B, leading to damage to normal tissues.

1-Substituted 2-nitroimidazoles are known to be selectively retained in poorly vascularized hypoxic tumor tissue by reductive metabolism to electrophiles.[9-14] As part of a program of synthesis and evaluation of nitroimidazoles in the treatment of cancer,[15-20] we have proposed that compounds containing 10-12 boron atoms linked to 2-nitroimidazole would form a useful method of concentrating boron in solid tumors. Derivatives of 1,2-dicarba-*closo*-dodecaborane(12) ("carborane", Figure 1) were selected for linkage to 2-nitroimidazole in view of their good chemical stability relative to other boron clusters and their predicted metabolic inertness. Prior to our first preliminary communication,[21] no synthesis of a nitroimidazole-carborane had been published in the journal literature, although reports of a short-chain nitroimidazole-*nido*-carborane[22] and a short-chain nitroimidazole-*meta*-carborane[23] have been made in proceedings of conferences.

In a previous paper,[20] we reported the 1,3-dipolar cycloaddition of a variety of nitroimidazole-alkene and -alkyne dipolarophiles with the highly stable 1,3-dipole, 4-(carboranylmethoxy)benzonitrile oxide. Although this strategy was successful in linking the potentially oxidizing 2-nitroimidazole with the carborane to produce the required nitroimidazole-carboranes, these latter materi-

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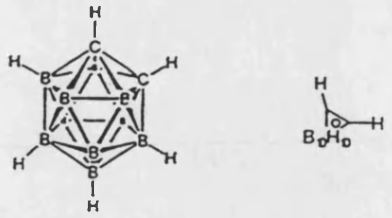
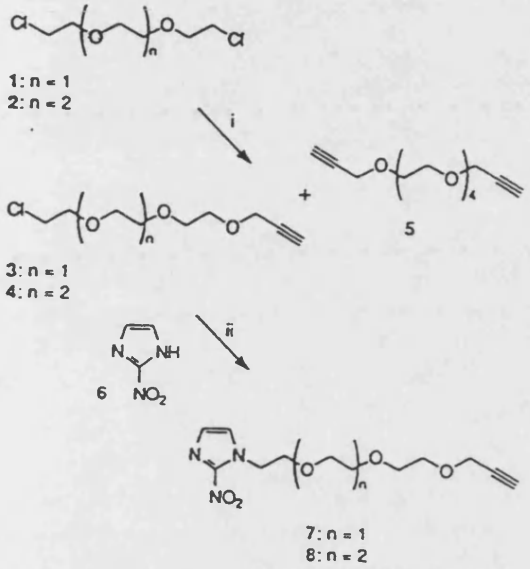


Figure 1. Representations of the structure of 1,2-dicarba-*closo*-dodecaborane(12) ("carborane"). All boron atoms have one hydrogen atom attached but some have been omitted from the diagram for clarity.

Scheme 1. Synthesis of the Polyether-Linked Nitroimidazole–Alkynes 7 and 8[a]



[a] Reagents and conditions: (i) $HC{\equiv}CCH_2OH$/NaH/DMF/130 °C; (ii) KOBu^t/DMF/120 °C.

als were insufficiently soluble in aqueous media to permit satisfactory evaluation of their tumor-localizing ability. Cycloadditions of alternative aliphatic carborane-nitrile oxides without the lipophilic aromatic ring have therefore been investigated and are reported here.

Synthesis of NitroimidazoleCarboranes

To provide nitroimidazole–alkynes with water-solubilizing oxyethylene units, the reaction sequences shown in Scheme 1 were adopted. As we reported previously,[20] treatment of 1,2-bis(2-chloroethoxy)ethane (1) with 1 equiv of the alkoxide of propargyl alcohol in hot DMF gave a good yield of the monoalkyne 3. Similar treatment of the higher oligomer 2 afforded the longer-chain monoalkyne 4 in moderate yield, along with a small quantity of the bis-alkyne 5. The yield of this bis-alkyne was increased in experiments in which an excess of alkoxide was employed. Modification of our previous conditions[20] for the alkylation of the potassium salt of 2-nitroimidazole (6) with 3 facilitated an improvement in the yield of the nitroimidazole–alkyne 7 from 43 to 66%. The analogous alkylation of 6 with 4 gave the longer-chain nitroimidazole–alkyne 8 with three ethylenedioxy units.

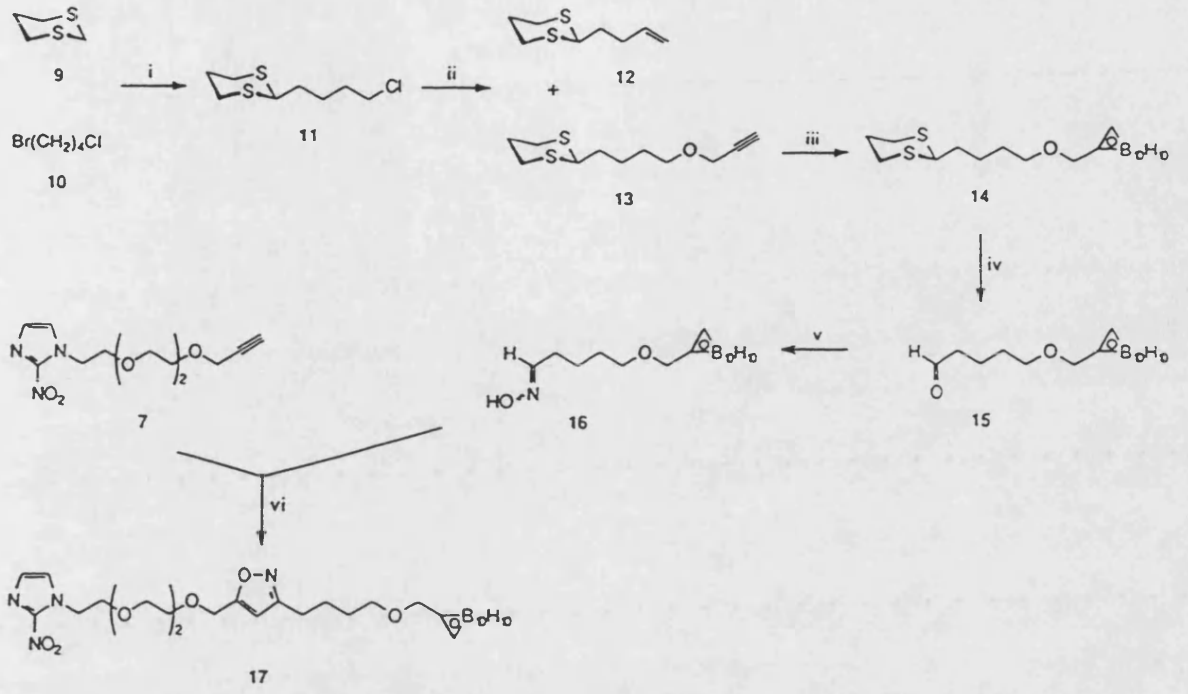
Construction of the oximes, precursors of the corresponding aliphatic carborane–nitrile oxides, was more challenging. Scheme 2 shows a sequence of model experiments to test the feasibility of use of the aliphatic carborane-nitrile oxides. 1,3-Dithiane (9) was lithiated and the anion was alkylated with bromochlorobutane (10), giving exclusively the known[24] (chlorobutyl)dithiane (11) in high yield. Substitution of chloride by propargyl alkoxide at elevated temperature in dimethylformamide gave the required dithiane–alkyne 13. This was accompanied by an apparently unavoidable elimination reaction of some of the substrate chlorobutyldithiane to furnish significant amounts of the butenyldithiane 12. This side reaction is probably due to direct E2 elimination caused by the strongly basic alkoxide. The dithiane–carborane 14 was formed in respectable yield in the usual way[20] by prolonged treatment of alkyne 13 with decaborane(14) ($B_{10}H_{14}$) in boiling acetonitrile, a Lewis base. Thus our use[20] of dithioacetal as a protecting group for aromatic aldehydes against the ravages of this potent reductant can be extended to analogous synthons for aliphatic aldehydes. However, mercury(II)-catalyzed deprotection, which was so successful for the aromatic case,[20] failed completely for the aliphatic dithioacetal, giving only unidentifiable products of degradation. Deprotection was, however, effected by the *N*-bromosuccinimide/silver ion method, although rapid quench with thiosulfate was needed to prevent degradation of the aldehyde 15 under these oxidizing conditions. The oxime 16 was formed as an equimolar mixture of geometrical isomers, as revealed by ^{1}H NMR. Unlike its aromatic counterpart,[20] the corresponding nitrile oxide was not isolable but formation by oxidation of 16 with sodium hypochlorite and reaction *in situ* in a two-phase aqueous dichloromethane system with the nitroimidazole–alkyne 7 gave the model nitroimidazole–isoxazole–carborane 17 in good yield.

On the basis of this successful demonstration of the 1,3-dipolar cycloaddition of a nitroimidazole–alkyne with an aliphatic carborane–nitrile oxide, attention was turned to the incorporation of potentially water-solubilizing multiple OCH_2CH_2O units in the carborane arm of the target structures (Scheme 3). With the aim of introducing two ether oxygens into this chain, 1,3-dithiane (9) was lithiated and alkylated with bis(2-chloroethyl) ether (18) in tetrahydrofuran, giving the ether-linked (haloalkyl)dithiane 19 in good yield, despite the usually lower reactivity of 3-oxaalkyl halides. Substitution then afforded the desired bis-ether-linked dithiane–alkyne 21. As predicted by the model tetramethylene system, elimination was a serious problem, giving the enol ether 20 in 36% yield. Clearly, the alkoxide is sufficiently basic even to remove the proton adjacent to ether oxygen to initiate E2 elimination. Conversion of the alkyne 21 to the dithiane–carborane 22 with decaborane(14) was followed by deprotection with *N*-bromosuccinimide/silver nitrate to furnish the carborane aldehyde 23. This aldehyde was found to be unstable to prolonged storage and was treated immediately with hydroxylamine in anhydrous ethanol, giving an equimolar mixture of *Z* and *E* stereoisomers of the carborane–oxime 24 virtually quantitatively.

With this bis-ether-linked carborane–oxime 24 now available, dipolar cycloaddition with the nitroimidazole alkynes 7 and 8 with three and four ether groups, respectively, was investigated. Oxidative elimination of the mixture of stereoisomers of 24 with sodium hypochlorite gave the corresponding nitrile oxide which was allowed to react in situ with alkynes 7 and 8. The yield of the shorter-chain nitroimidazole–isoxazole–carborane

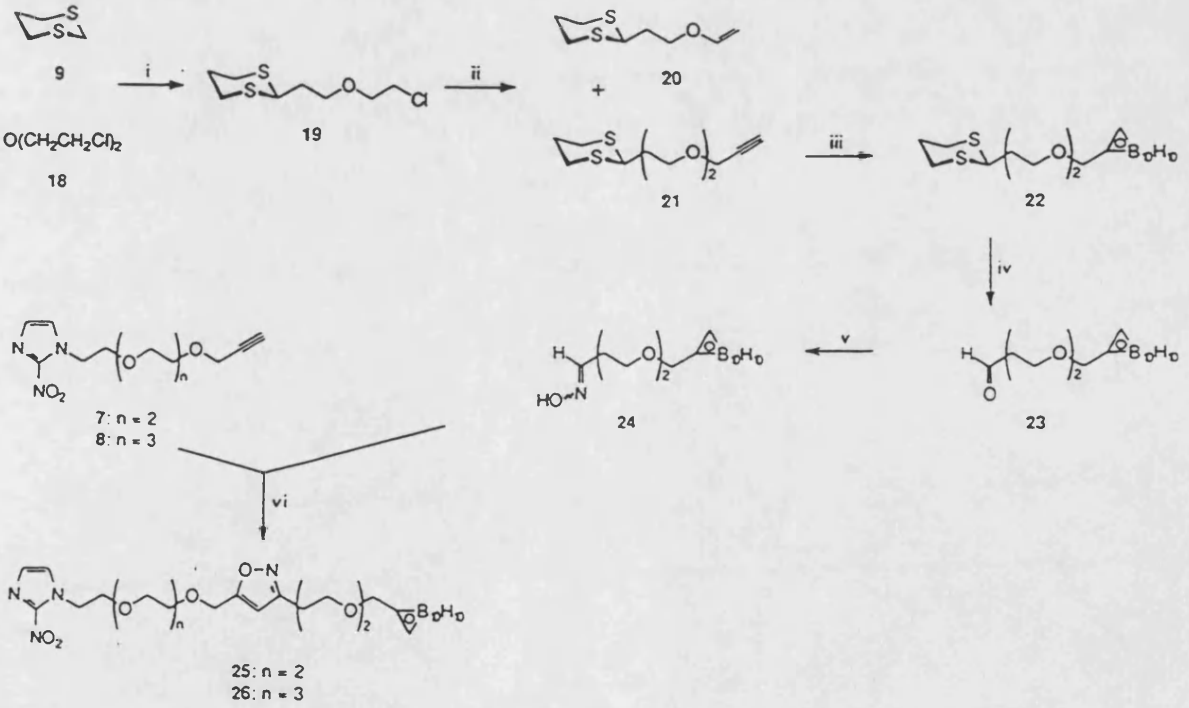
(24) Seebach, D.; Jones, N. R.; Corey, E. J. *J. Org. Chem.* 1968, *33*, 300.

Scheme 2. Model Experimental Sequence for the Synthesis of the Nitroimidazole–Isoxazole–Carborane 17[a]



[a] Reagents and conditions: (i) NaH/THF/−78 °C — −20 °C; (ii) HC≡CCH$_2$OH/NaH/DMF/100 °C; (iii) $B_{10}H_{14}$/MeCN/reflux; (iv) *N*-bromosuccinimide/$AgNO_3$/water/MeCN; (v) $NH_2OH{\cdot}HCl/Na_2CO_3$/EtOH; (vi) NaOCl/water/$CH_2Cl_2$.

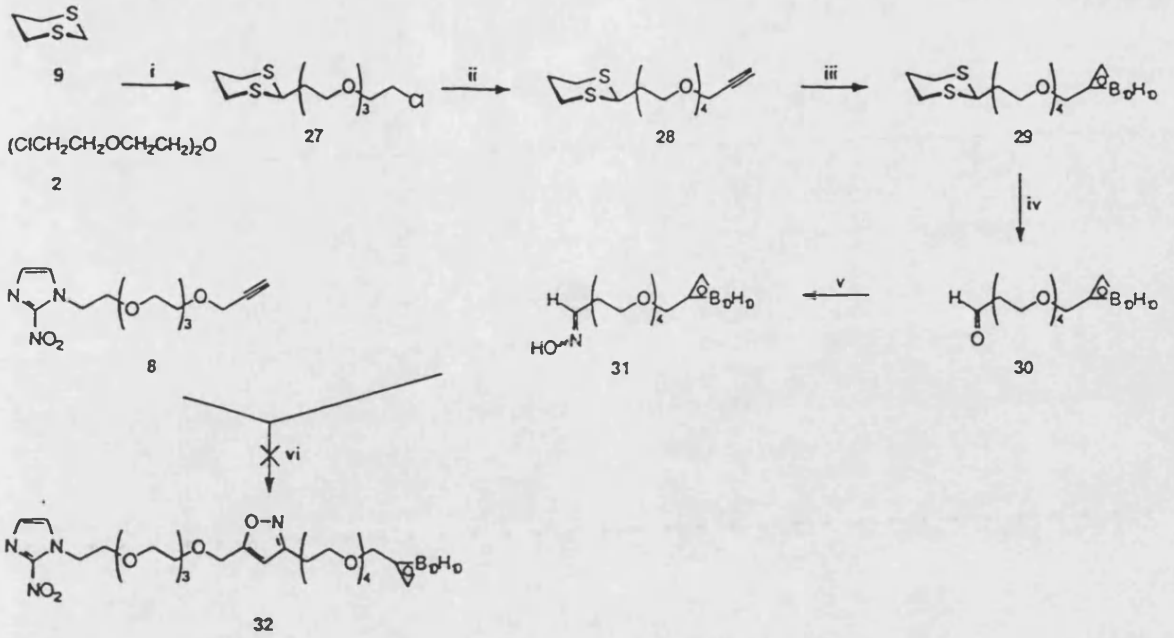
Scheme 3. Synthesis of Target Polyether-Linked Nitroimidazole–Isoxazole–Carboranes 25 and 26[a]



[a] Reagents and conditions: (i) NaH/THF/−78 °C — −20 °C; (ii) NaH/HC≡CCH$_2$OH/DMF/120 °C; (iii) $B_{10}H_{14}$/MeCN/reflux; (iv) *N*-bromosuccinimide/$AgNO_3$/water/MeCN; (v) $NH_2OH{\cdot}HCl/Na_2CO_3$/EtOH; (vi) NaOCl/water/$CH_2Cl_2$.

25 was not able to be raised above a disappointing 13% in experiments where reaction times and concentrations were varied. On the other hand, the longer-chain analogue 26 was formed in 36% yield, based on the oxime 24, which corresponds to 90% when compared with the amount of nitroimidazole–alkyne 8 not recovered from the reaction mixture. Alternative oxidants, such as chloramine-T, were less effective. The oxime was completely consumed in all experiments and no material containing the nitrile oxide group could be detected amongst the products of the reaction. These observations, coupled with the high recovery of the alkyne, point

Scheme 4. Attempted Synthesis of Extended Polyether–Linked Nitroimidazole–Isoxazole–Carborane 32[a]



[a] Reagents and conditions: (i) NaH/THF/−78 °C — −20 °C; (ii) NaH/HC≡CCH$_2$OH/DMF/120 °C; (iii) $B_{10}H_{14}$/MeCN/reflux; (iv) *N*-bromosuccinimide/$AgNO_3$/water/MeCN; (v) NH_2OH·HCl/Na_2CO_3/EtOH.

to the instability of the intermediate nitrile oxide being the cause of the relatively modest yields of isoxazoles.

Since the physical properties (polarity, solubility, etc.) of nitroimidazole–carboranes **25** and **26**, with five and six ether oxygens, respectively, were considerably improved with respect to the aryl analogues previously reported,[20] synthesis of the extended analogue **32**, with eight ether oxygens, was attempted (Scheme 4). Alkylation of the anion of 1,3-dithiane (**9**) with dichloro compound **2** at ambient temperature gave the (trioxachloroalkyl)dithiane **27**. The decreased reactivity of the polyether chain, as compared with polymethylene analogues, was reflected in the low (25%) yield of the dithiane–alkyne **28** through substitution with propynoxide ion in hot dimethylformamide. The formation of the carborane **29** under the usual conditions (decaborane(14), refluxing acetonitrile) was, however, unaffected and proceeded relatively efficiently. Deprotection was again effected by bromination, giving carborane–aldehyde **30** which was converted immediately to its oxime **31**. However, the sensitivity of the cycloaddition reaction to the structure of the oxime (and hence the nitrile oxide) was revealed in the failure to produce isolable quantities of the desired nitroimidazole–isoxazole–carborane **32** upon treatment of **31** with the nitroimidazole–alkyne **8** and sodium hypochlorite in aqueous dichloromethane. Much unreacted alkyne **8** was detected by TLC, although the oxime **31** was consumed, suggesting that the nitrile oxide derived from **31** was particularly unstable.

Conclusions

The present work has demonstrated the feasibility of extending the known[20] 1,3-dipolar cycloaddition of carborane-substituted aryl nitrile oxides with nitroimidazole–alkynes to the use of the considerably less stable aliphatic carborane–nitrile oxides. The synthesis of the nitroimidazole–carborane **26** in adequate yield based on the relatively expensive starting materials 2-nitroimidazole (overall yield 36%) and decaborane(14) (overall yield 18%) has enabled sufficient quantities of this relatively polar carborane to be available for biological evaluation.

Experimental Section

The solvent for NMR spectra was $CDCl_3$. The external standard for ^{11}B NMR was BF_3Et_2O. Purity for all novel compounds was assessed by elemental microanalysis or by ^1H NMR; the ^1H NMR spectra for compounds **4**, **5**, **7**, **8**, **12**-**17**, **19**-**31** are available as supplementary material. Brine refers to saturated aqueous NaCl. DMF refers to dimethylformamide; THF refers to tetrahydrofuran. Dry EtOH was dried by distillation from $Mg(OEt)_2$ immediately before use. Solutions in organic solvents were dried with anhydrous $MgSO_4$. Distillations were effected using a Kugelrohr apparatus and bps refer to the temperature of the oven. The stationary phase for chromatography was silica gel. Solvents were evaporated under reduced pressure. 3-(2-(2-(2-Chloroethoxy)ethoxy)ethoxy)propyne (**3**) was prepared as previously described by us.[16]

3-(2-(2-(2-(2-Chloroethoxy)ethoxy)ethoxy)ethoxy)propyne (4) and Bis(2-(2-(prop-2-ynyloxy)ethoxy)ethyl) Ether (5). Sodium hydride (1.20 g, 50 mmol) was stirred with propyn-3-ol (2.80 g, 50 mmol) in dry DMF for 30 min. Bis(2-(2-chloroethoxy)ethyl) ether **2** (11.55 g, 50 mmol) was added dropwise and the mixture was stirred at 130 °C for 2 h. The solvent was evaporated and the residue, in CH_2Cl_2, was washed with water and dried. The evaporation residue was subjected to chromatography (pentane/Et_2O 3:1, 2:1, and then 1:1). From the first fraction was recovered **2** (3.25 g, 23%). From the second fraction was obtained the monoalkyne **4** (3.67 g, 29%) as a colorless oil: IR ν 2120 cm^{-1}; NMR δ_H 2.43 (1 H, t, J = 2.4 Hz), 3.6–3.8 (16 H, m), 4.21 (2 H, d, J = 2.4 Hz); mass spectrum (CI) m/z 251.1050 (M + H) ($C_{11}H_{20}{}^{35}ClO_4$ requires 251.1050). From the third fraction was isolated the bis-alkyne **5** (950 mg, 7%) as a colorless oil: IR ν 2130 cm^{-1}; NMR δ_H 2.44 (2 H, t, J = 2.4 Hz), 3.67 (8 H, s), 3.69 (8 H, m), 4.21 (4 H, d, J = 2.4 Hz); mass spectrum (CI) m/z 271.1545 (M + H) ($C_{11}H_{20}{}^{35}ClO_4$ requires 271.1545).

2-Nitro-1-(2-(2-(2-(propyn-3-yloxy)ethoxy)ethoxy)ethyl)imidazole (7). 2-Nitroimidazole (**6**) (570 mg, 5 mmol) was stirred at 130 °C with KOBu^t (560 mg, 5 mmol) in DMF (10 mL) for 1 h then cooled to 80 °C. KI (50 mg) and the alkyne **3** (1.03 g, 5 mmol) were added and the mixture was stirred at 120 °C for 16 h. The solvent was evaporated and the residue

was treated with water (10 mL) and then extracted with ether. Chromatography (CH_2Cl_2) of the evaporation residue gave the nitroimidazole–alkyne **7** (930 mg, 66%) as a pale yellow oil: IR ν 2120 cm^{-1}; NMR δ_H 2.45 (1 H, t, J = 2.4 Hz), 3.60 (4 H, s), 3.6–3.7 (4 H, m), 3.86 (2 H, t, J = 4.9 Hz), 4.20 (2 H, d, J = 2.4 Hz), 4.63 (2 H, t, J = 4.9 Hz), 7.14 (1 H, s), 7.29 (1 H, s); mass spectrum (CI) m/z 284.1246 (M + H) ($C_{12}H_{18}N_3O_5$ requires 284.1246).

2-Nitro-1-(2-(2-(2-(2-(propyn-3-yloxy)ethoxy)ethoxy)ethoxy)ethyl)imidazole (8). 2-Nitroimidazole (**6**) (450 mg, 4 mmol) was stirred at 120 °C with KOBu^t (450 mg, 4 mmol) in DMF (5 mL) for 15 min then cooled to 80 °C. NaI (40 mg) and the alkyne **4** (1.00 g, 4 mmol) were added and the mixture was stirred at 120 °C for 16 h. The evaporation residue was treated with water (20 mL) and was extracted with CH_2Cl_2. Chromatography (CH_2Cl_2/Et_2O 5:1) of the evaporation residue gave the nitroimidazole–alkyne **8** (520 mg, 40%) as a pale yellow oil: NMR δ_H 2.44 (1 H, t, J = 2.4 Hz), 3.6–3.7 (12 H, m), 3.86 (2 H, t, J = 5.0 Hz), 4.19 (2 H, d, J = 2.4 Hz), 4.63 (2 H, t, J = 5.0 Hz), 7.13 (1 H, s), 7.36 (1 H, s); mass spectrum (CI) m/z 328.1509 (M + H) ($C_{14}H_{22}N_3O_6$ requires 328.1509).

2-(4-Chlorobutyl)-1,3-dithiane (11). Butyllithium (2 M in hexanes, 20 mL, 40 mmol) was added to 1,3-dithiane (**9**) (4.80 g, 40 mmol) in THF (100 mL) and the mixture was stirred for 1 h, before being cooled to −78 °C. 1-Bromo-4-chlorobutane (**10**) (8.58 g, 60 mmol) was added and the mixture was stirred at −20 °C for 18 h. The evaporation residue, in CH_2Cl_2, was washed with water and dried. Distillation gave 2-(4-chlorobutyl)-1,3-dithiane (**11**) (6.88 g, 82%) as a colorless liquid: $bp_{0.5}$ 170 °C (lit.[24] $bp_{0.17}$ 110 °C); NMR δ_H 1.6–1.9 (7 H, m), 2.11 (1 H, m), 2.85 (4 H, m), 3.54 (2 H, t, J = 6.5 Hz), 4.06 (1 H, t, J = 6.5 Hz).

2-(But-1-en-4-yl)-1,3-dithiane (12) and 2-(4-(Propyn-3-yloxy)butyl)-1,3-dithiane (13). Propyn-3-ol (220 mg, 4 mmol) was added to sodium hydride (96 mg, 4 mmol) in dry DMF (10 mL) and the mixture was stirred for 30 min. The (chloroalkyl)dithiane **11** (840 mg, 4 mmol) was added and the mixture was heated at 100 °C for 2 h. The evaporation residue, in CH_2Cl_2, was washed with water and dried. The solvent was evaporated and the residue was subjected to chromatography (pentane/CH_2Cl_2 4:1 and then 3:1). From the earlier fraction was obtained 2-(but-1-en-4-yl)-1,3-dithiane (**12**) (240 mg, 34%) as a colorless oil: NMR δ_H 1.85 (2 H, br q, J = 7 Hz), 1.87 (1 H, m), 2.15 (1 H, m), 2.27 (2 H, br q, J = 7 Hz), 2.85 (4 H, m), 4.04 (1 H, t, J = 7.0 Hz), 5.02 (1 H, br d, J = 10 Hz), 5.07 (1 H, br d, J = 17 Hz), 5.80 (1 H, ddt, J = 17.0, 10.3, 6.6 Hz); mass spectrum (EI) m/z 174.0537 (M) ($C_8H_{14}S_2$ requires 174.0537). From the later fraction was isolated 2-(4-(propyn-3-yloxy)butyl)-1,3-dithiane (**13**) (480 mg, 52%) as a colorless oil: NMR δ_H 1.6–2.0 (7 H, m), 2.12 (1 H, m), 2.42 (1 H, t, J = 2.4 Hz), 2.85 (4 H, m), 3.52 (2 H, t, J = 6.2 Hz), 4.05 (1 H, t, J = 6.9 Hz), 4.13 (2 H, d, J = 2.4 Hz); mass spectrum (EI) m/z 230.0799 (M) ($C_{11}H_{18}OS_2$ requires 230.1799).

2-(4-(1,2-Dicarba-*closo*-dodecaboran(12)-1-ylmethoxy)-butyl)-1,3-dithiane (14). Decaborane(14) ($B_{10}H_{14}$) (210 mg, 1.7 mmol) was stirred with dry MeCN (10 mL) for 3 h. The dithiane–alkyne **13** (400 mg, 1.7 mmol) was added and the mixture was boiled under reflux for 3 d. The solvent was evaporated. Chromatography (pentane/CH_2Cl_2 10:1) gave the carborane-dithiane **14** (290 mg, 49%) as a white solid: mp 72–74 °C; NMR δ_H 2.3 (10 H, br q, J_{B-H} = 150 Hz), 1.6–2.2 (8 H, m), 2.85 (4 H, m), 3.46 (2 H, t, J = 5.3 Hz), 3.83 (2 H, s), 3.98 (1 H, br s), 4.05 (1 H, t, J = 6.8 Hz). Anal. ($C_{11}H_{28}B_{10}OS_2$) C, H.

4-(1,2-Dicarba-*closo*-dodecaboran(12)-1-ylmethoxy)-pentanal (15). *N*-Bromosuccinimide (1.25 g, 7 mmol) and $AgNO_3$ (1.36 g, 8 mmol) in MeCN (16 mL) and water (4 mL) were added to the carborane–dithiane **14** (480 mg, 1.4 mmol) in MeCN (16 mL) and water (4 mL), and the mixture was stirred for 10 min. Saturated aqueous Na_2SO_3 (2.0 mL) was added, followed after 1 min by saturated aqueous Na_2CO_3 (2.0 mL), followed after 1 min by brine (2.0 mL). The suspension was filtered through Celite. The filtrate was treated with brine (20 mL) and extracted with CH_2Cl_2. Chromatography (CH_2Cl_2) of the evaporation residue gave the carborane–aldehyde **15** (260 mg, 71%) as a colorless oil: IR ν 1725, 2590 cm^{-1}; NMR δ_H 2.2 (10 H, br q, J_{B-H} = 150 Hz), 1.63 (4 H, m), 2.48 (2 H, dt, J = 1.4, 6.8 Hz), 3.47 (2 H, t, J = 5.9 Hz), 3.84 (2 H, s), 3.94 (1 H, br s), 9.78 (1 H, t, J = 1.4 Hz); mass spectrum (CI) m/z 259.2701 (M + H) ($C_8H_{23}{}^{10}B_2{}^{11}B_8O_2$ requires 259.2701)

5-(1,2-Dicarba-*closo*-dodecaboran(12)-1-ylmethoxy)-pentanal Oxime (16). The carborane–aldehyde **15** (260 mg, 1 mmol) was stirred with hydroxylamine hydrochloride (350 mg, 5 mmol) and anhydrous Na_2CO_3 (270 mg, 2.5 mmol) in dry EtOH (10 mL) for 24 h. Further portions of hydroxylamine hydrochloride (350 mg, 5 mmol) and Na_2CO_3 (270 mg, 2.5 mmol) were added, and stirring was continued for a further 18 h. The solvent was evaporated and the residue, in CH_2Cl_2, was washed with water and dried. Chromatography (CH_2Cl_2) of the evaporation residue gave a mixture of *E* and *Z* isomers of the carborane–oxime **16** (221 mg, 81%) as a colorless oil: IR ν 2590, 3300 (br) cm^{-1}; NMR δ_H 1.21 (4 H, m), 2.2 (10 H, br q, J_{B-H} = 150 Hz), 2.26 (1 H, m), 2.41 (1 H, m), 3.47 (2 H, m), 3.84 (2 H, s), 3.97 (1 H, br s), 6.72 (0.5 H, t, J = 5.5 Hz), 7.42 (0.5 H, t, J = 5.8 Hz), 8.9 (0.5 H, br), 10.5 (0.5 H, br); NMR δ_C 22.51, 22.90, 28.54, 28.80, 28.93, 29.03, 57.57, 71.49, 71.68, 72.59, 151.50, 152.06; mass spectrum (FAB negative ion) m/z 273.2635 (M − H) ($C_8H_{22}{}^{10}B^{11}B_9NO_2$ requires 273.2617), 272.2654 (M − H) ($C_8H_{22}{}^{10}B_2{}^{11}B_8NO_2$ requires 272.2653).

1-(2-(2-(2-((3-(4-(1,2-Dicarba-*closo*-dodecaboran(12)-1-ylmethoxy)butyl)isoxazol-5-yl)methoxy)ethoxy)ethoxy)-ethyl)-2-nitroimidazole (17). The carborane–oxime **16** (140 mg, 0.5 mmol) and the nitroimidazole–alkyne **7** (170 mg, 0.6 mmol) in CH_2Cl_2 (5 mL) were stirred with aqueous NaOCl (8% available chlorine, 2 mL) for 2 d. Water (5 mL) was added and the mixture was extracted with CH_2Cl_2. The evaporation residue was subjected to chromatography (Et_2O and then EtOAc). From the Et_2O fraction was recovered the nitroimidazole–alkyne **7** (90 mg, 53%). From the EtOAc fraction was obtained the nitroimidazole–carborane **17** (130 mg, 47%) as a colorless gum: NMR δ_H 1.65 (4 H, m), 2.2 (10 H, br q, J_{B-H} = 150 Hz), 2.67 (2 H, t, J = 7.2 Hz), 3.48 (2 H, t, J = 6.0 Hz), 3.6–3.7 (8 H, m), 3.84 (2 H, s), 3.85 (2 H, t, J = 5.0 Hz), 3.96 (1 H, br s), 4.61 (2 H, s), 4.61 (2 H, t, J = 5.0 Hz), 6.11 (1 H, s), 7.10 (1 H, d, J = 1.0 Hz), 7.25 (1 H, d, J = 1.0 Hz); mass spectrum (FAB positive ion) m/z 557.3760 (M + H) ($C_{20}H_{39}{}^{11}B_{10}N_4O_7$ requires 557.3749).

2-(2-(2-Chloroethoxy)ethyl)-1,3-dithiane (19). Butyllithium (2 M in hexanes, 50 mL, 100 mmol) was added to 1,3-dithiane (**9**) (12.0 g, 100 mmol) in THF (250 mL) at −78 °C and the mixture was stirred at this temperature for 1 h. Bis-(2-chloroethyl) ether (**18**) (14.3 g, 100 mmol) was added and the mixture was stirred at −20 °C for 16 h. The solvent was evaporated and the residue, in CH_2Cl_2, was washed with water and dried. Distillation gave the (chloroalkyl)dithiane **19** (13.77 g, 61%) as a colorless liquid: $bp_{0.1}$ 160 °C; NMR δ_H 1.89 (1 H, m), 2.03 (2 H, dt, J = 7.0, 6.1 Hz), 2.12 (1 H, m), 2.80–2.95 (4 H, m), 3.60–3.75 (6 H, m), 4.23 (1 H, t, J = 7.0 Hz); mass spectrum m/z (CI) 227.0331 (M + H) ($C_8H_{16}{}^{35}ClOS_2$ requires 227.0331).

2-(2-(Ethenyloxy)ethyl)-1,3-dithiane (20) and 2-(2-(2-(Propyn-3-yloxy)ethoxy)ethyl)-1,3-dithiane (21). A stirred suspension of sodium hydride (1.51 g, 63 mmol) in dry DMF (60 mL) was treated with propyn-3-ol (3.47 g, 62 mmol) in dry DMF (40 mL) and the mixture was stirred for 30 min. NaI (100 mg) and the dithiane **19** (14.04 g, 62 mmol) were added and the mixture was stirred at 120 °C for 3 h. The evaporation residue, in CH_2Cl_2, was washed with water and dried. The evaporation residue was subjected to chromatography (pentane/CH_2Cl_2 1:1, and then CH_2Cl_2). From the earlier fraction was obtained 2-(2-(ethenyloxy)ethyl)-1,3-dithiane (**20**) (4.21 g, 36%) as a colorless oil: IR ν 1625 cm^{-1}; NMR δ_H 1.91 (1 H, m), 2.11 (3 H, m), 2.86 (4 H, m), 3.86 (2 H, t, J = 6.1 Hz), 4.02 (1 H, dd, J = 6.9, 2.1 Hz), 4.21 (1 H, t, J = 7 Hz), 4.21 (1 H, dd, J = 14.3, 2.1 Hz), 6.46 (1 H, dd, J = 14.3, 6.9 Hz); mass spectrum (EI) m/z 190.0486 (M) ($C_8H_{14}OS_2$ requires 190.0486). From the CH_2Cl_2 fraction was obtained the dithiane–alkyne **21** (6.20 g, 47%) as a colorless oil: NMR δ_H 1.88 (1 H, m), 2.04 (2 H, q, J = 7.0 Hz), 2.10 (1 H, m), 2.44 (1 H, t, J = 2.4 Hz), 2.85 (4 H, m), 3.65 (6 H, m), 4.21 (1 H, t, J = 7.0 Hz), 4.22 (2 H, d, J = 2.4 Hz); mass spectrum (EI) m/z 246.0748 (M) ($C_{11}H_{18}O_2S_2$ requires 246.0748).

2-(2-(2-(1,2-Dicarba-*closo*-dodecaboran(12)-1-yl)ethoxy)-ethyl)-1,3-dithiane (22). Decaborane(14) (305 mg, 2.5 mmol) was stirred with MeCN (10 mL) for 3 h. The dithiane–alkyne 21 (620 mg, 2.5 mmol) was added and the mixture was boiled under reflux for 3 d. The solvent was evaporated. Chromatography (pentane/CH_2Cl_2 1:1) gave the carborane-dithiane 22 (550 mg, 60%) as a colorless oil: IR ν 2600 cm^{-1}; NMR δ_H 1.2–3.2 (10 H, br q, J_{B-H} = 150 Hz), 1.86 (1 H, m), 1.98 (2 H, dt, J = 7.1, 5.9 Hz), 2.14 (1 H, m), 2.52 (2 H, t, J = 5.6 Hz), 2.87 (4 H, m), 3.51 (2 H, t, J = 5 Hz), 3.53 (2 H, t, J = 5.9 Hz), 4.08 (1 H, br s), 4.15 (1 H, t, J = 7.1 Hz); NMR δ_C 25.83, 30.12, 36.39, 43.88, 57.60, 67.33, 69.99, 71.28, 72.14, 72.80; mass spectrum m/z (FAB positive ion) 367.2493 (M + H) ($C_{11}H_{29}{}^{11}B_{10}O_2S_2$ requires 367.2540).

3-(2-(1,2-Dicarba-*closo*-dodecaboran(12)-1-ylmethoxy)-ethoxy)propanal (23). The carborane-dithiane 22 was treated with *N*-bromosuccinimide and $AgNO_3$ in aqueous MeCN, as for the synthesis of 16, to give the carborane–aldehyde 23 (610 mg, 86%) as a yellow oil: IR ν 1725, 2590 cm^{-1}; NMR δ_H 2.2 (10 H, br q, J_{B-H} = 150 Hz), 2.68 (2 H, dt, J = 2.2, 6.7 Hz); 3.6 (4 H, m), 3.79 (2 H, t, J = 6.7 Hz), 3.96 (2 H, s), 4.02 (1 H, br s), 9.89 (1 H, d, J = 2.2 Hz). This material was used immediately without further purification.

3-(2-(1,2-Dicarba-*closo*-dodecaboran(12)-1-ylmethoxy)-ethoxy)propanal Oxime (24). The carborane–aldehyde 23 (450 mg, 1.6 mmol) in dry EtOH (100 mL) was treated with anhydrous Na_2CO_3 (850 mg, 8 mmol) and $HONH_2$·HCl (1.11 g, 16 mmol) for 40 h. The evaporation residue, in CH_2Cl_2, was washed with water and dried. The solvent was evaporated to give the carborane–oxime 24 (460 mg, 97%) as a pale yellow gum: IR ν 2590, 3340 (br) cm^{-1}; NMR δ_H 1.4–2.7 (10 H, br q, J_{B-H} = 150 Hz), 2.48 (1 H, q, J = 6 Hz), 2.66 (1 H, q, J = 5.5 Hz), 3.55–3.67 (6 H, m), 3.94 (1 H, s), 3.95 (1 H, s), 4.13 (1 H, br s), 5.1 (0.5 H, br), 6.81 (0.5 H, t), 7.46 (0.5 H, t, J = 5.8 Hz), 8.0 (0.5 H, br); mass spectrum (CI) m/z 290.2759 (M + H) ($C_8H_{23}{}^{10}B_2{}^{11}B_8NO_3$ requires 290.2759).

1-(2-(2-(2-((3-(2-(2-(1,2-Dicarba-*closo*-dodecaboran(12)-1-ylmethoxy)ethoxy)ethyl)isoxazol-5-yl)methoxy)ethoxy)-ethoxy)ethyl)-2-nitroimidazole (25). The carborane–oxime 24 (40 mg, 0.14 mmol) and the nitroimidazole–alkyne 7 (40 mg, 0.14 mmol) in CH_2Cl_2 (2 mL) were stirred with aqueous NaOCl (8% available chlorine, 2.5 mL) for 3 d. The mixture was extracted with CH_2Cl_2. The evaporation residue was subjected to chromatography (Et_2O and then EtOAc). From the EtOAc fraction was obtained the nitroimidazole–carborane 25 (10 mg, 13%) as a colorless gum: IR ν 2590 cm^{-1}; NMR δ_H 2.2 (10 H, br q, J_{B-H} = 150 Hz), 2.93 (2 H, t, J = 6.6 Hz), 3.60–3.75 (14 H, m), 3.86 (2 H, t, J = 5.2 Hz), 3.92 (2 H, s), 4.13 (1 H, br s), 4.60 (4 H, m), 6.15 (1 H, s), 7.10 (1 H, br s), 7.25 (1 H, br s); mass spectrum (FAB positive ion) m/z 571.3788 (M + H) ($C_{20}H_{39}{}^{10}B_2{}^{11}B_8N_4O_8$ requires 571.3771).

1-(2-(2-(2-(2-((3-(2-(2-(1,2-Dicarba-*closo*-dodecaboran-(12)-1-ylmethoxy)ethoxy)ethyl)isoxazol-5-ylmethoxy)-ethoxy)ethoxy)ethoxy)ethyl)-2-nitroimidazole (26). The carborane–oxime 24 (130 mg, 0.45 mmol) and the nitroimidazole–alkyne 8 (150 mg, 0.45 mmol) in CH_2Cl_2 (5 mL) were stirred with aqueous NaOCl (8% available chlorine, 2 mL) for 2 d. Brine was then added and the mixture was extracted with CH_2Cl_2. The evaporation residue was subjected to chromatography (Et_2O and then EtOAc). From the Et_2O fraction was recovered the nitroimidazole–alkyne 8 (90 mg, 60%). From the EtOAc fraction was obtained the nitroimidazole–carborane 26 (100 mg, 36%) as a pale yellow gum: IR ν 2590cm^{-1}; NMR δ_H 2.2 (10 H, br q, J_{B-H} = 150 Hz), 2.91 (2 H, t, J = 6.4 Hz), 3.60–3.75 (18 H, m), 3.86 (2 H, t, J = 5.0 Hz), 3.92 (2 H, s), 4.15 (1 H, br s), 4.62 (4 H, m), 6.18 (1 H, s), 7.11 (1 H, d, J = 0.8 Hz), 7.28 (1 H, d, J = 0.8 Hz); NMR δ_B −15.18 (4 B, m), −13.66 (2 B, d, J_{B-H} = 150 Hz), −11.14 (2 B, d, J_{B-H} = 147 Hz), −6.90 (1 B, d, J_{B-H} = 150 Hz), −5.04 (1 B, d, J_{B-H} = 150 Hz); mass spectrum (FAB negative ion) m/z 613.3872 (M − H) ($C_{22}H_{41}{}^{10}B_2{}^{11}B_8N_4O_9$ requires 613.3877).

2-(2-(2-(2-(2-Chloroethoxy)ethoxy)ethoxy)ethyl)-1,3-dithiane (27). Butyl lithium (2 M in hexanes, 20.5 mL, 41 mmol) was added to 1,3-dithiane (9) (4.80 g, 40 mmol) in dry THF (80 mL) at −78 °C under N_2 and the mixture was stirred at this temperature for 1 h. Bis(2-(2-chloroethoxy)ethyl) ether (2) (9.24 g, 40 mmol) was added and the mixture was stirred at −20 °C for 20 h. The solvent was evaporated and the residue, in CH_2Cl_2, was washed with water and dried. Chromatography (pentane/CH_2Cl_2 1:1, and then CH_2Cl_2) gave the (chloroalkyl)dithiane 27 (5.16 g, 49%) as a pale yellow oil: NMR δ_H 1.89 (1 H, m), 2.0–2.2 (3 H, m), 2.85 (4 H, m), 3.6–3.8 (14 H, m), 4.20 (1 H, t, J = 7.0 Hz); mass spectrum (FAB positive ion) m/z 314.0772 (M) ($C_{12}H_{23}{}^{35}ClO_3S_2$ requires 314.0777).

2-(2-(2-(2-(2-(Propyn-3-yloxy)ethoxy)ethoxy)ethoxy)-ethyl)-1,3-dithiane (28). Propyn-3-ol (560 mg, 10 mmol) was added to sodium hydride (240 mg, 10 mmol) in dry DMF (10 mL) and the mixture was stirred for 30 min. The chloroalkyl–dithiane 27 (3.14 g, 10 mmol) was added and the mixture was stirred at 120 °C for 3 h. The solvent was evaporated. The residue, in CH_2Cl_2, was washed with water and dried. Chromatography (CH_2Cl_2/Et_2O 20:1, and then 10:1) gave the dithiane–alkyne 28 (830 mg, 25%) as a pale yellow oil: IR ν 2120 cm^{-1}; NMR δ_H 1.8–2.2 (4 H, m), 2.44 (1 H, t, J = 2.4 Hz), 2.85 (4 H, m), 3.63 (14 H, m), 4.20 (1 H, t, J = 7.1 Hz), 4.21 (2 H, d, J = 2.4 Hz); mass spectrum (FAB positive ion) m/z 335.1317 (M + H) ($C_{15}H_{27}O_4S_2$ requires 335.1351), 334.1272 (M) ($C_{15}H_{26}O_4S_2$ requires 334.1273).

2-(2-(2-(2-(2-(1,2-Dicarba-*closo*-dodecaboran(12)-1-yl-methoxy)ethoxy)ethoxy)ethoxy)ethyl)-1,3-dithiane (29). Decaborane(14) (120 mg, 1 mmol) was stirred in dry MeCN (10 mL) for 3 h. The dithiane–alkyne 28 (330 mg, 1 mmol) was added and the mixture was boiled under reflux for 3 d. Chromatography (CH_2Cl_2/Et_2O 20:1) of the evaporation residue gave the carborane-dithiane 29 (260 mg, 58%) as a pale yellow gum: IR ν 2590 cm^{-1}; NMR δ 1.85 (1 H, m), 2.04 (2 H, q, J = 7.0 Hz), 2.08 (1 H, m), 2.2 (10 H, br q, J_{B-H} = 150 Hz), 2.85 (4 H, m), 3.64 (14 H, m), 3.96 (2 H, s), 4.10 (1 H, br s), 4.19 (1 H, t, J = 7.0 Hz); mass spectrum (FAB positive ion) m/z 452.3060 (M + H) ($C_{15}H_{36}{}^{10}B_2{}^{11}B_8O_4S_2$ requires 452.3058).

3-(2-(2-(2-(1,2-Dicarba-*closo*-dodecaboran(12)-1-yl-methoxy)ethoxy)ethoxy)ethoxy)propanal (30). The carborane–dithiane 29 was treated with *N*-bromosuccinimide and $AgNO_3$ in aqueous MeCN, as for the synthesis of 15, except that the eluant for chromatography was CH_2Cl_2/Et_2O, to give the carborane–aldehyde 30 (74%) as a pale yellow gum: IR ν 1725, 2590 cm^{-1}; NMR δ_H 2.2 (10 H, br q, J_{B-H} = 150 Hz), 2.70 (2 H, dt, J = 6.2, 1.8 Hz), 3.62 (12 H, m), 3.83 (2 H, t, J = 6.2 Hz (becomes s on decoupling at δ 2.70)), 3.96 (2 H, s), 4.10 (1 H, br s), 9.80 (1 H, t, J = 1.8 Hz); mass spectrum (FAB positive ion) m/z 345.3070 (M + H − H_2O) ($C_{12}H_{29}{}^{10}B_2{}^{11}B_8O_4$ requires 345.3069); mass spectrum (CI) m/z 307.2913 (100%) (M + H − H_2C=CHCHO) ($C_9H_{27}{}^{10}B_2{}^{11}B_8O_4$ requires 307.2913).

3-(2-(2-(2-(1,2-Dicarba-*closo*-dodecaboran(12)-1-yl-methoxy)ethoxy)ethoxy)ethoxy)propanal Oxime (31). The carborane–aldehyde 30 was treated with $HONH_2$·HCl and Na_2CO_3, as for the synthesis of 16, except that chromatography was omitted, to give an equimolar mixture of geometrical isomers of the carborane–oxime 31 (80%) as a colorless gum: IR ν 2590, 3350 (br) cm^{-1}; NMR δ_H 2.2 (10 H, br q, J_{B-H} = 150 Hz), 2.49 (1 H, q, J = 6.2 Hz), 2.66 (1 H, q, J = 6.2 Hz), 3.64 (14 H, m), 3.96 (2 H, s), 4.12 (1 H, br s), 6.85 (0.5 H, t, J = 6.2 Hz), 7.48 (0.5 H, t, J = 6.2 Hz), 8.0 (1 H, br); mass spectrum (FAB positive ion) $^{10}B/^{11}B$ isotope cluster centered at m/z 378 (M + H).

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Supplementary Material Available: Copies of ^1H NMR spectra of 4, 5, 7, 8, 12-17, and 19-31 (23 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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CHAPTER 4

THE METABOLISM OF TRIAZENE ANTITUMOR DRUGS

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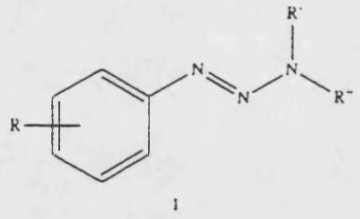
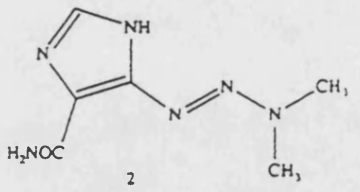
1. INTRODUCTION

1-Aryl-3,3-dialkyltriazenes (1, Fig. 1) are carcinogens (Preussmann et al., 1969a; Druckrey, 1913) and the dimethyl analogues (1; R′, R″ CH_3) show potent antineoplastic activity against rodent tumors (Clarke et al., 1955; Burchenal et al., 1956). Use of these potently biologically active compounds has also been claimed for such diverse applications as herbicides (Mazza et al., 1974), reagents for the determination of heavy metals (Popescu and Danet, 1983) and agents to render grain unpalatable to pests (Adams and Wright, 1964). Structure–antitumor activity studies on 1-aryl-3,3-dialkyltriazenes have shown that at least one *N*-methyl group is necessary for activity and that substitution in the phenyl ring has little effect on activity against the TLX5 lymphoma in mice (Audette et al., 1973; Connors et al., 1976). However, quantitative structure–activity relationships (QSAR) established by Hatheway et al. (1978) and by Trinajstic et al. (1989) have indicated a good correlation between antitumor activity and the presence of electron-donating substituents on the phenyl ring but that partition coefficient (expressed as log *P*) has little effect. Various attempts have been made to attach the 3,3,-dimethyltriazen-1-yl-phenyl 'warhead' to 'delivery' moieties such as 2,4-diaminopyrimidines (Bliss et al., 1987) and peptides (Ionescu et al., 1981). 3-Acyl-1-(2-chloroethyl)-3-methyltriazenes have also been claimed to be useful antineoplastic agents (Michejda and Smith, 1989). During the last 25 years, the role of metabolism in the mechanisms of the carcinogenicity and cytotoxicity of aryldialkyltriazenes has been studied extensively. However, many important aspects of these mechanisms remain enigmatic. One heteroaryldimethyltriazene, 5-(3,3-dimethyltriazen-1-yl)imidazole-4-carboxamide (dacarbazine, DTIC; 2, Fig. 1), is of moderate clinical usefulness in the treatment of disseminated melanoma, cancer of the colon and Hodgkin's



1



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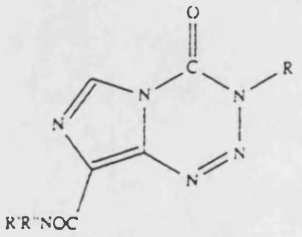

3: R = CH_2CH_2Cl; R′,R″ = H
4: R = CH_3; R′,R″ = H
5: R = CH_2CH_2Cl; R′,R″ = CH_2
6: R = CH_2CH_2Cl; R′ = CH_3; R″ = H

FIG. 1. Structures of arylalkyltriazenes (1), dacarbazine (2), mitozolomide (3), temozolomide (4) and analogues.

disease which is resistant to therapy with MOPP (a combination of mechlorethamine, vincristine, procarbazine and prednisone) (Loo et al., 1976; Spassova and Golovinsky, 1985). Some 1-aryl-3,3-dimethyltriazenes and 1-aryl-3-methyltriazenes also show strong antimetastatic activity which is probably mediated through xenogenization of the tumor cells, leading to attack by the immune system (Nardelli et al., 1984; Grill et al., 1987; Nifontov et al., 1988). Although DNA is generally taken to be the biochemical site of cytotoxic, carcinogenic and mutagenic action of triazenes, a correlation between inhibition of Ca^{2+}-activated ATPase and antimetastatic activity against the murine Lewis lung carcinoma has been noted (Nifontov et al., 1988). Curiously, most studies of the metabolism and mechanism of the antineoplastic action of dimethyltriazenes have been carried out using substituted phenyldimethyltriazenes rather than dacarbazine itself.

Recently, a number of cyclic arylmonoalkyltriazenes, the 3-alkyl-8-carbamoylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-ones (e.g. 3–6, Fig. 1) have been designed as potential therapeutic alternatives to dacarbazine (Stevens et al., 1984). They exhibit potent antineoplastic properties in rodents (Hickman et al., 1985; Fodstad et al., 1985). Mitozolomide (8-carbamoyl-3-(2-chloroethyl)-imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-one; 3) underwent clinical trial and was found to cause severe prothromocytopenia as a clinically unacceptable, dose-limiting toxicity (Harding et al., 1988). Temozolomide (8-carbamoyl-3-methylimidazo[5,1-*d*]-1,2,3,5-tetrazin-1(3H)-one; 3) is currently under clinical evaluation.

The chemical properties and therapeutic aspects of the 1-aryl-3,3-dialkyltriazenes and the chemical properties of the imidazotetrazinones have been reviewed extensively (Kolar, 1984; Spassova and Golovinsky, 1985; Threadgill, 1990). Hence, this chapter is principally confined to aspects of their metabolism, together with appropriate biomimetic chemistry.

In aqueous solution at physiological or mildly basic pH, 1-aryl-3,3-dialkyltriazenes are moderately stable. In contrast, acidic environments cause rapid reversal of the usual synthetic reaction (Gescher et al., 1981), giving the arenediazonium salt (7, Fig. 2) and the protonated secondary amine (8). This degradation yields potently electrophilic species and therefore this pathway could be considered to be the source of biologically reactive entities. However, no firm evidence has been put forward to support the contention that this reaction occurs at a significant rate under physiological conditions.

The oxidative metabolism of 1-aryl-3,3-dialkyltriazenes by hepatic microsomes in vitro was first reported in 1967 (Druckrey et al., 1967) and this study and subsequent investigations (e.g. Preussmann et al., 1969b; Kolar and Schlesiger, 1976a; Kolar and Wildschütte, 1982) have left little

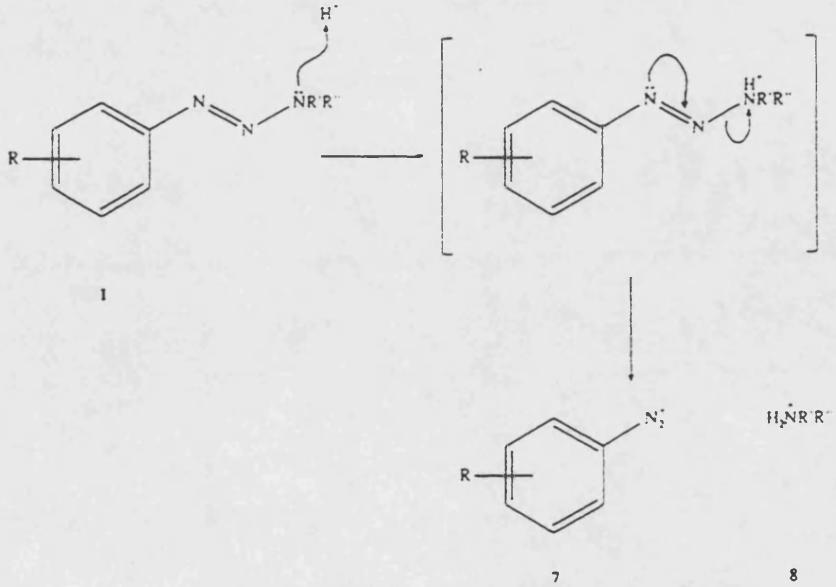

FIG. 2. Mechanism of acid-catalyzed decomposition of aryldialkyltriazenes by reversal of the general synthetic pathway.

doubt that oxidative *N*-dealkylation is a metabolic pathway essential for the biological properties of these compounds.

2. OXIDATIVE METABOLISM OF 1-ARYL-3,3-DIALKYLTRIAZENES AT THE ALKYL GROUPS

In the study by Preussmann et al. (1969b), 3,3-dimethyl-1-phenyltriazene (9, Fig. 3) was incubated with rat liver microsomes and NADPH. Formaldehyde was reported to be a metabolite and the authors suggested that the other product of this oxidation was 3-methyl-1-phenyltriazene (11, Fig. 3). Monoalkyltriazenes such as this are powerful directly-acting alkylating agents (13, Fig. 4) and are known to be carcinogens (Vaughan and Stevens, 1978). They react spontaneously with nucleophiles, giving the corresponding arylamine (14), molecular nitrogen and the alkylated nucleophile (15). It can be assumed that it is this reaction mechanism which is operative in biological alkylations involving monoalkyltriazenes rather than a reaction which has been claimed to involve the 'methyl carbonium ion' (16), as the latter is very unlikely to occur in aqueous biological media, although the methyl diazonium ion has been put forward as the ultimate mutagen derived from 3-acyl-1,3-dimethyltriazenes in *Salmonella* (Smith et al., 1986). Deuterium labelling studies have shown that diazoalkanes (17) are unlikely to be involved as intermediates in these alkylations (Lown and Singh, 1981). There is good evidence that the reaction between monomethyltriazenes and bionucleophiles such as nucleic acids can occur in vivo (Preussmann et al., 1969b; Kleihues et al., 1979; Margison et al., 1979). Therefore, the oxidative metabolic pathway (Fig. 3) has been postulated to be responsible for the generation of the ultimate cytotoxin which is responsible for the carcinogenicity and, possibly, the antitumor activity of aryldialkyltriazenes.

Many of the studies of the metabolism of aryldialkyltriazenes conducted in the wake of the original report by Preussmann et al. (1969b) used colorimetric determination of the aldehyde generated by hydroxylation of the *N*-alkyl group mediated by cytochromes *P*450 enzymes and subsequent hydrolysis/elimination. In that study, some 20–80% of the alkyl groups were oxidized to aldehydes, depending on the chemical nature of the alkyl moieties (on the basis that complete

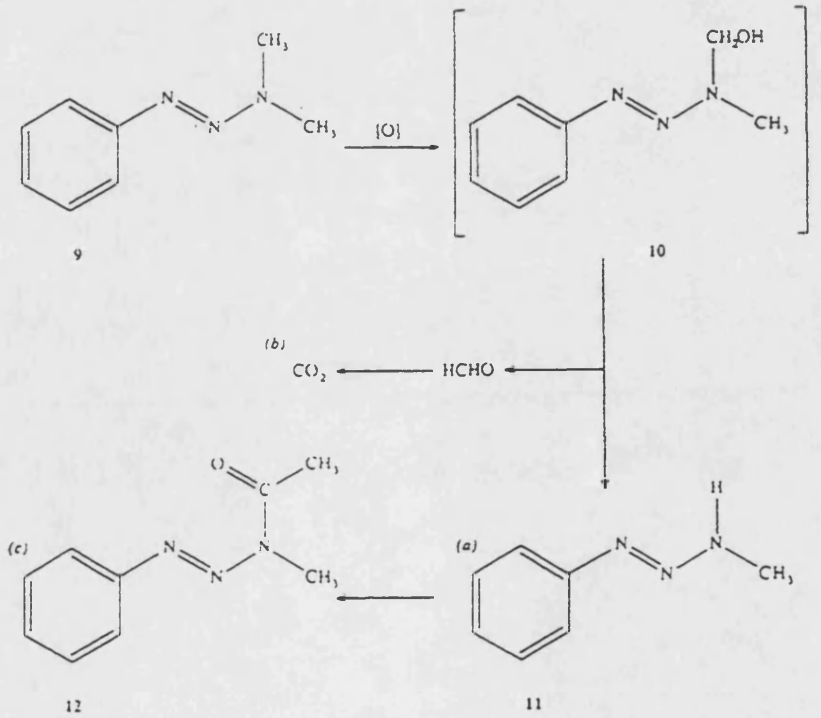

FIG. 3. Metabolism of 3,3-dimethyl-1-phenyltriazene (9) by rodent hepatic microsomes in vitro. (a) Preussmann and von Hodenberg, 1969. (b) Kleihues et al., 1976. (c) Pool, 1979b.

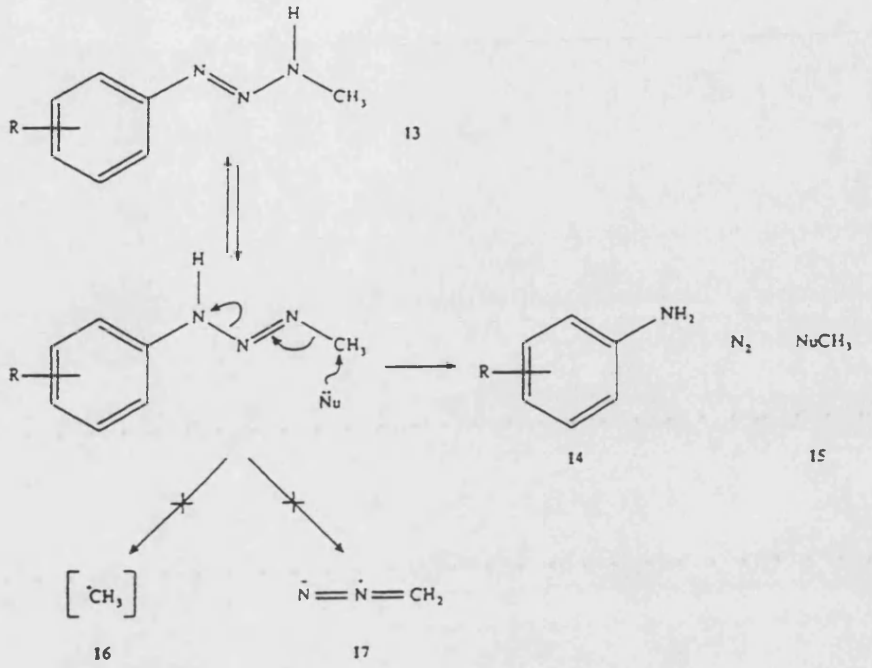

FIG. 4. Postulated mechanisms of methylation by monomethyltriazenes. Nu = H_2O, nucleophilic sites on DNA, proteins, etc.

removal of one of the two alkyl groups equals 100%). A later study showed that incubation of four (haloaryl)dimethyltriazenes and the unsubstituted 3,3-dimethyl-1-phenyltriazene with microsomes led to the loss of between 35 and 122% of the methyl groups as formaldehyde (calculated on the above basis) (Pool, 1979a). These results imply some oxidation of the second methyl group. 3,3-Dimethyl-1-phenyltriazene yielded the lowest and 1-(2,4,6-trichlorophenyl)3,3-dimethyltriazene (18, Fig. 5) gave the highest extent of oxidation at N-CH_3 (Pool, 1979a), although Kolar and Habs (1984) have reported that the presence of halogen substituents on the benzene ring decreases the extent of this metabolism. There have been several other studies on the effect of the nature of the (substituted)aryl group on the extent of metabolism at the N-CH_3 groups of aryldimethyltriazenes; some of these studies give conflicting results and conclusions (Preussmann et al., 1969b; Audette et al., 1973; Giraldi et al., 1975). Inhibition of this metabolism by metabolic products has been

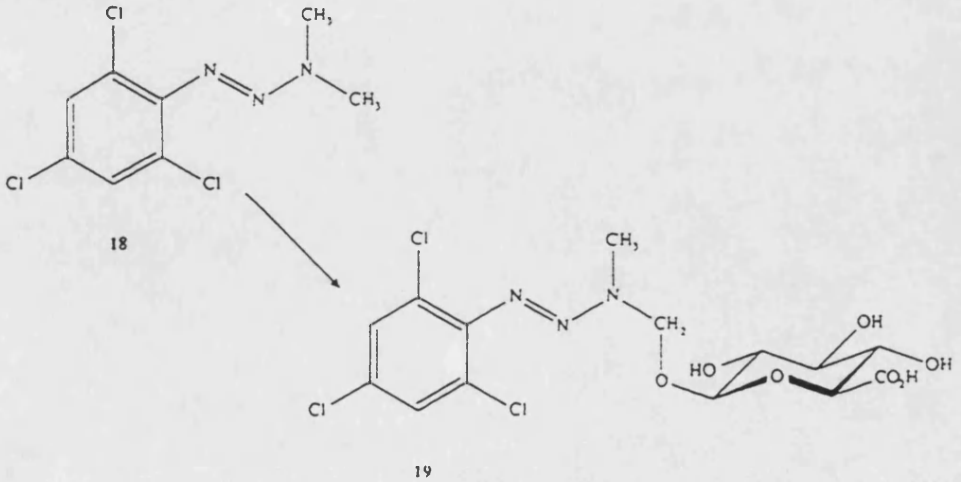

FIG. 5. Metabolic oxidation/conjugation of 3,3-dimethyl-1-(2,4,6-trichlorophenyl)triazene (18) in the mouse.

noted (Giraldi et al., 1975). The ultimate fate of some 40% of the radioactivity administered as 3,3-di[^{14}C]methyl-1-phenyltriazene to rats was reported to be exhalation as $^{14}CO_2$ by Kleihues et al. (1976). These workers and Hradec and Kolar (1985) also observed the formation of 7-[^{14}C]methylguanine and other methylated DNA and RNA bases. *N*-Hydroxy-*N*-methyltriazenes appear not to be substrates for oxidative metabolism (Preussmann et al., 1969b).

Work by Stevens et al. (1979) using chemical biomimetic oxidation of related triazenes in which the two alkyl groups are part of a heteroalicyclic ring has shown facile oxidation to nitrogen, although the chemical products were devoid of antitumor activity. The Udenfriend system has been used as a chemical model for the oxidative metabolism of 1-aryl-3,3-dimethyltriazenes to reactive methylating agents (Matrka et al., 1988).

Unambiguous and direct identification of a monoalkyltriazene as a metabolite of a 1-aryl-3,3-dimethyltriazene was first reported by Farina et al. (1982); 1-(4-acetylphenyl)-3-methyltriazene (21, Fig. 6), together with 4-aminoacetophenone (22), was found as a metabolite of 1-(4-acetylphenyl)-3,3-dimethyltriazene (20) both in the plasma of mice in vivo and in 9000 × *g* fractions of mouse liver homogenate in vitro. A more indirect observation of a monomethyltriazene as a metabolite of a dimethyltriazene has been made in that one of the metabolites found in incubations of rodent hepatic microsomes with 3,3-dimethyl-1-phenyltriazene was 3-acetyl-3-methyl-1-phenyltriazene (12, Fig. 3) (Pool, 1979b). Presumably, the formation of this *N*-acyltriazene involves the monomethyltriazene (11) as a metabolic intermediate which is subsequently acetylated.

The immediate metabolic precursors of 1-aryl-3-methyltriazenes, the 1-aryl-3-(hydroxymethyl)-3-methyltriazenes (26, Fig. 7A), have been synthesized (Gescher et al., 1978; Juillard et al., 1980). However, such carbinolamines have not yet been identified unambiguously as metabolites of aryldimethyltriazenes. At first sight, it is difficult to envisage how such compounds could be more selectively cytotoxic than their decomposition products, the monomethyltriazenes. Certain *N*-(hydroxymethyl)amines and *N*-(hydroxymethyl)amides related to *N*-(hydroxymethyl)-*N*-methyltriazenes have the ability to form, or can be in equilibrium with, electrophilic, potentially toxic imines or iminium ions, either themselves or after conjugation; the chemical features which predispose *N*-(hydroxymethyl) compounds to undergo such reactions under physiological conditions have been reviewed (Overton et al., 1985). Such an elimination reaction (Fig. 7A) could, on paper, be a mechanism by which hydroxymethyltriazenes exert toxicity in a manner different from that which renders monomethyltriazenes cytotoxic (Hemens et al., 1984; Soloway et al., 1983). However, a chemical and kinetic study of the reactions of 3-(hydroxymethyl)-3-methyl-1-(pyridin-3-yl)triazene with amine bases to give 3-methyl-1-(pyridin-3-yl)triazene as the sole product (Cheng et al., 1985) and the failure of the treatment of 1-aryl-3-(hydroxymethyl)-3-methyltriazenes with sodium cyanoborohydride, an iminium trapping agent, to yield 3,3-dimethyltriazenes (M. D. Threadgill, unpublished results) suggest that this is not an electrophilic reaction pathway open to *N*-(hydroxymethyl)triazenes under physiological conditions. Nevertheless, it has been shown that a reactive iminium is formed during chemical reactions of acetate and benzoate esters of *N*-(hydroxymethyl)triazenes (28 and 29, respectively, Fig. 7B) with nucleophiles. Presumably the enhanced leaving-group ability of the carboxylate leads to this increased electrophilicity (Hemens et al., 1984; Hemens and Vaughan, 1986; Iley et al., 1987a), although Vaughan et al. (1987) postulate a direct non-ionic S_N2 reaction of 28 with azide ion to give a new type of prodrug of monomethyltriazenes, which does not require oxidative metabolism. However, such acylation of a *N*-(hydroxymethyl)triazene has yet to be shown in biochemical experiments. Methyleneiminium triazenes are also formed during reactions of *N*-(hydroxymethyl)triazenes with nucleophiles under acidic conditions (Iley et al., 1987b, 1988).

The rather unstable nature of *N*-(hydroxymethyl)-*N*-methyltriazenes (Vaughan et al., 1984) has been claimed to mitigate against their role as the ultimate antineoplastic species derived from dimethyltriazenes. Nevertheless, the possibility that they are important as precursors of monomethyltriazenes with a transport function cannot be excluded, particularly if they are stabilized as the corresponding glucuronide acetate conjugate. It is pertinent in this context to note that Kolar and Carubelli (1979) identified 1-{[3-methyl-1-(2,4,6-trichlorophenyl)triazen-1-1]methyl}-β-D-glucuronic acid (19, Fig. 5) as a metabolite in the urine of mice which had received 3,3-dimethyl-1-(2,4,6-trichlorophenyl)triazene (18) and the authors suggested that this conjugate may, indeed, carry the monomethyltriazene to the target tissue. Metabolic conjugation of triazenes

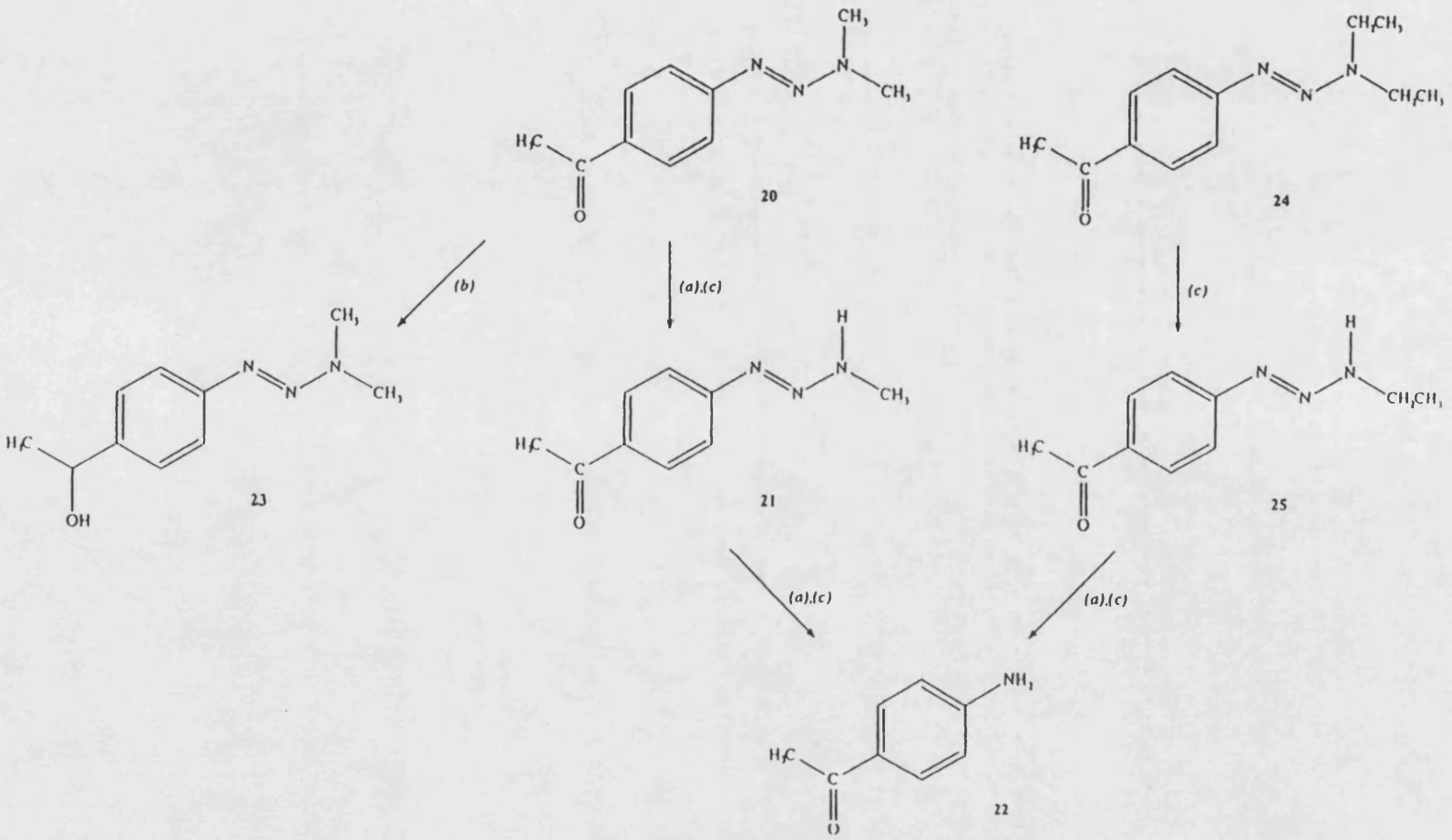

FIG. 6. Oxidative and reductive metabolic pathways demonstrated for 1-(4-acetylphenyl)triazenes (16) and (24). (a) Farina et al., 1982. (b) Farina et al., 1983. (c) Farina et al., 1986.

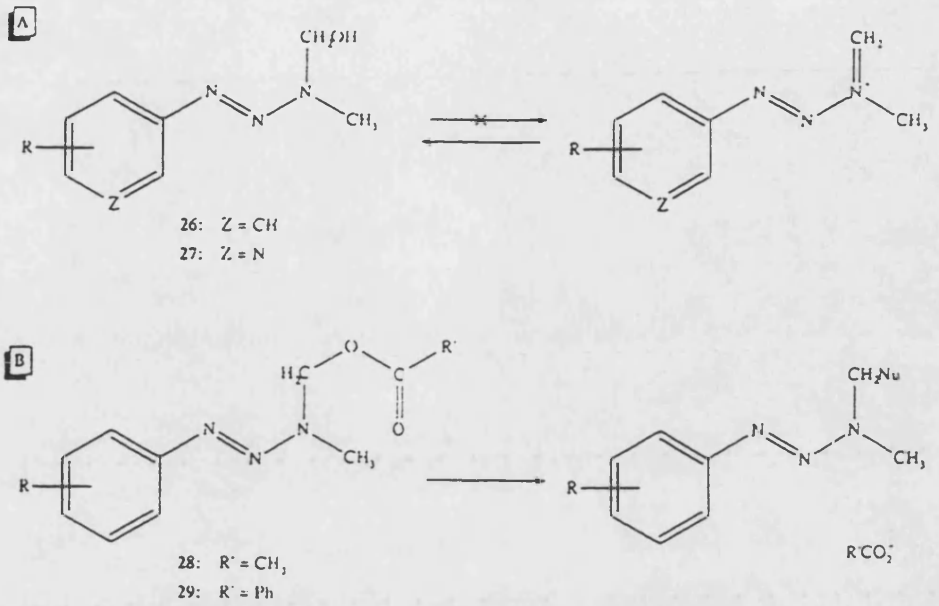

FIG. 7. (A) Electrophilic iminium ions from *N*-(hydroxymethyl)triazenes. (B) Electrophilic reactivity of *N*-(acyloxymethyl)triazenes.

to glutathione and to sulfate has yet to be reported, although a putative glutathione conjugate has been synthesized chemically (Iley et al., 1988).

Monomethyltriazenes and 3-(hydroxymethyl)-3-methyltriazenes generated as metabolites of dimethyltriazenes could be considered as substrates for further metabolism. Indeed, aniline is a metabolite of 3,3-dimethyl-1-phenyltriazene in vitro in amounts equimolar to the amount of formaldehyde formed (Druckrey et al., 1967; Preussmann et al., 1969b) and Toren et al. (1984) have detected methanol formed metabolically from 1-aryl-3-methyltriazenes and from a 1-aryl-3-(hydroxymethyl)-3-methyltriazene. When further oxidative metabolism is considered, it should be noted that analogous *N*-(hydroxymethyl)benzamides are subject to further metabolic oxidation at the methylene group yielding the corresponding *N*-(hydroxymethyl)benzamide which is excreted by the mouse in the urine (Ross et al., 1983). *N*-(Hydroxymethyl)triazenes, however, have not yet been reported to undergo this metabolic oxidation to afford the corresponding *N*-formyltriazenes which are highly reactive entities (Ignasiak et al., 1975; Threadgill and Gledhill, 1986; Butler et al., 1987), although *N*-formyltriazenes are the principal products of oxidation of *N*-methyltriazenes and *N*-hydroxymethyltriazenes by a chemical model for cytochromes P450 (Iley and Ruecroft, 1988). Some evidence for a second biological oxidation has been obtained in experiments in which the fate of 1-(4-acetylphenyl)3-methyltriazene (21, Fig. 6) in suspensions of murine hepatocytes was investigated (Farina et al., 1983). The monomethyltriazene was consumed in this medium at a rate significantly more rapid than in suspensions of nonviable cells. The chemical nature of the products of this metabolic reaction are completely unknown.

Metabolic oxidation at the nitrogen atoms of the triazene group of an arylmonoalkyltriazene has been observed only once, in that 3-(4-carboxy-3-fluorophenyl)-1-methyltriazene-1-oxide was revealed by ^{19}F NMR spectroscopy to be a urinary metabolite of the corresponding dimethyltriazene, 3-fluoro-4-(3,3-dimethyltriazen-1-yl)benzoic acid (Vaughan and Wilman, 1991).

3. METABOLISM REMOTE FROM THE TRIAZENE MOIETY

1-Aryl-3,3-dimethyltriazenes undergo metabolism also at sites remote from the triazene moiety, i.e. in the aryl part of the molecule or involving transformation of functional groups attached thereto. Kolar and Schlesiger (1976b) reported indirect evidence for such metabolites in that the

major urinary metabolite derived from 3,3-dimethyl-1-phenyltriazene in rats was found to be 4-aminophenol (30) with smaller amounts of the 3-amino and 2-amino isomers (31 and 32, respectively, Fig. 8). These authors also proposed ring-hydroxylated triazenes as metabolites of this substrate but only after chemical coupling with 1-(ethylamino)naphthalene to give the azo dyes 1-(ethylamino)-4(4-hydroxybenzeneazo)naphthalene (35) and 1-(ethylamino)-4-(2-hydroxybenzeneazo)naphthalene (36). This derivatization involves the loss of N^3 of the triazene (with its attached groups) and thus the urinary triazenes 33 and 34 may have been 3,3-dimethyl, 3-methyl or even 3-(hydroxymethyl)-3-methyl compounds. A more direct example of metabolism remote from the triazene moiety with confirmed retention of the N^3 substituents is that the ketone carbonyl group of 1-(4-acetylphenyl)-3,3-dimethyltriazene (20) was reduced by preparations of murine hepatic tissue in vitro to give 1-[4-(1-hydroxyethyl)phenyl]-3,3-dimethyltriazene (29, Fig. 6). Interestingly, this material retained an antitumor activity similar to that of the parent drug (Farina et al., 1983). The principal urinary metabolites of the experimental drug, 4-(3,3-dimethyl-1-triazenyl)benzoic acid (1; R = 4-CO_2H) have been found to be the glucuronide and glycine conjugates of the parent dimethyltriazene; the corresponding monomethyltriazene and its derivatives were not observed (Benfenati et al., 1989).

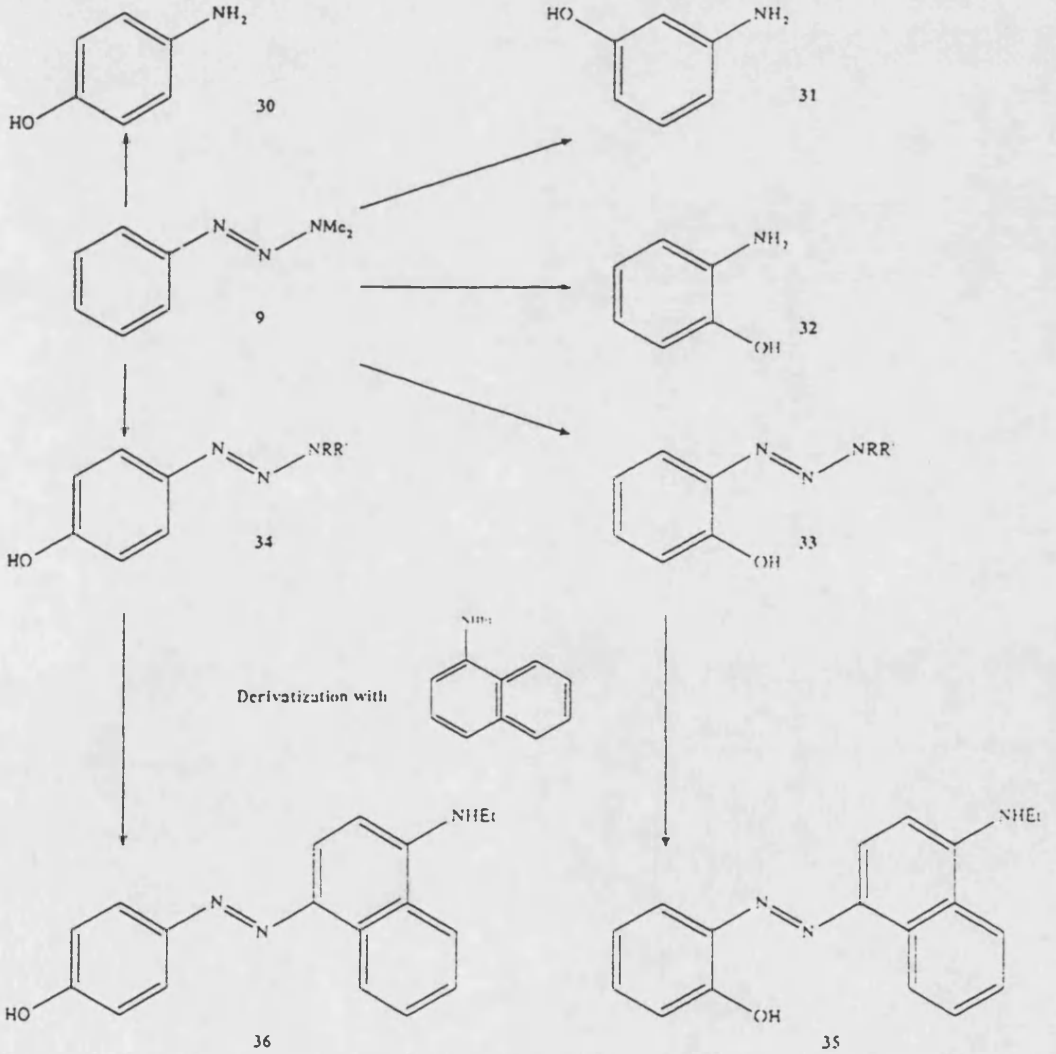

FIG. 8. Aryl-hydroxylated metabolites of 3,3-dimethyl-1-phenyltriazene (9) in the mouse. R = CH_3; R' = H, CH_2OH, CH_3.

4. METABOLIC FATE OF 1-(IMIDAZOL-5-YL)-3-ALKYLTRIAZENES

The major urinary metabolite of dacarbazine (2) in man is 5-aminoimidazole-4-carboxamide (AIC: 39, Fig. 9) (Householder and Loo, 1969; Skibba et al., 1970a,b; Breithaupt et al., 1982). AIC has also been identified as a product of metabolism of dacarbazine in vitro in incubations with mouse liver microsomes (Hill, 1975) and with human and animal tumor tissue (Gerulath and Loo, 1972; Mizuno and Humphrey, 1972). From the mechanistic point of view, it is difficult to envisage how AIC could be formed by a metabolic pathway other than one implicating the intermediate formation of 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MTIC: 38), followed by the tautomerism noted by Hooper and Vaughan (1981), amongst others, and subsequent hydrolysis. Many workers have reported that metabolism is required for the antitumor activity of dacarbazine to be manifest (Skibba et al., 1969a,b; Skibba and Bryan, 1971; Preussmann et al., 1969a). 5-[3-(Hydroxymethyl)-3-methyltriazen-1-yl]imidazole-4-carboxamide (HMTIC: 37, Fig. 9), the metabolic precursor of MTIC, has been characterized as a urinary metabolite of dacarbazine in rats (Kolar et al., 1980). Surprisingly, this hydroxymethyl compound was reported to be more stable than MTIC in polar solvents. This observation led to the suggestion that HMTIC may act as a transport form for MTIC, the postulated ultimate cytotoxic species derived from dacarbazine. After the manner of 3-methyl-1-phenyltriazene, MTIC decomposes spontaneously in aqueous media to form AIC (39) and was capable of alkylating biological nucleophiles such as DNA (Mizuno and Decker, 1976). The pharmacokinetic studies published so far on dacarbazine (Householder and Loo, 1971; Loo et al., 1968, 1976; Skibba et al., 1969a,b; Breithaupt et al., 1982) have not provided information on concentrations in plasma or tissue of metabolically generated HMTIC or MTIC. Therefore, the pharmacokinetic data available on dacarbazine is of limited value in helping to relate the efficacy or toxicity of this drug to the concentrations of cytotoxic or biologically inactive metabolites in body fluids and tissues. The major shortcoming of most of the pharmacokinetic data reported in the literature is the analytical procedure employed, a colorimetric method which determines the total amount of compounds with intact triazene linkages and therefore does not discriminate between dacarbazine, HMTIC and MTIC. The plasma elimination half-life of such species after

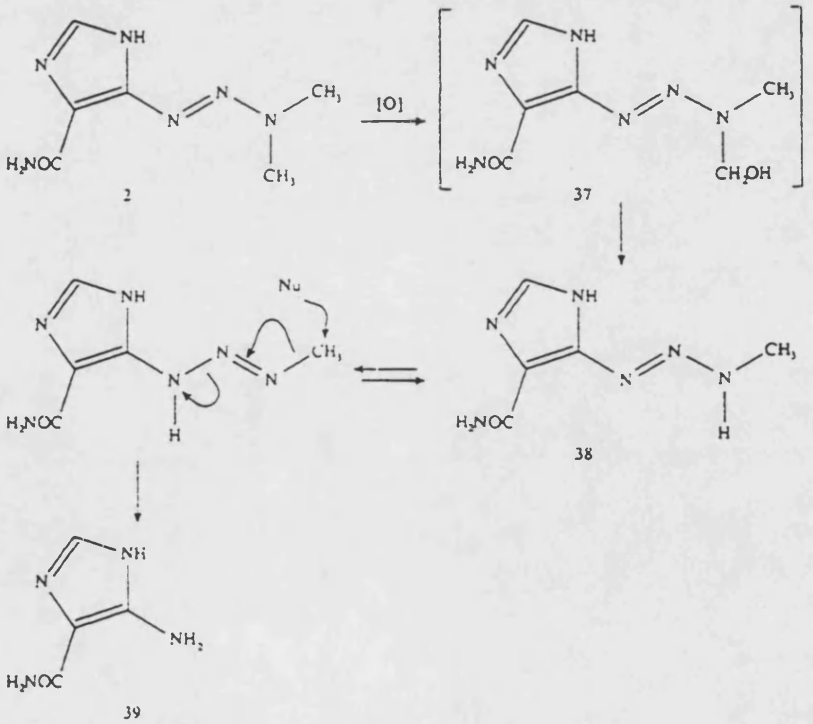

FIG. 9. Metabolic activation of dacarbazine (2) to the cytotoxin MTIC (38). Nu = H_2O, nucleophilic sites on DNA, proteins, etc

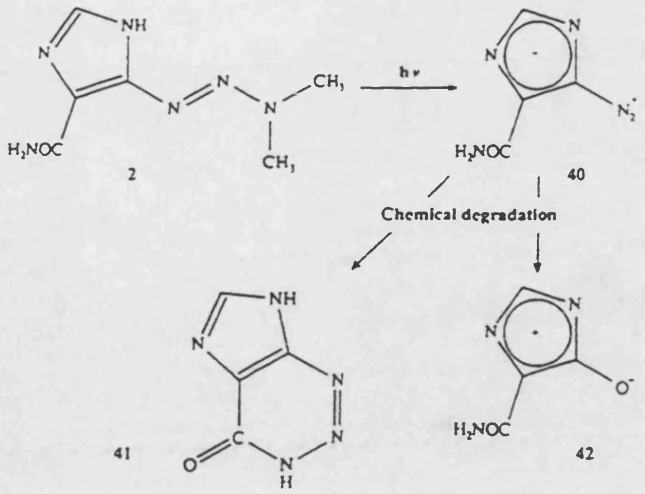

FIG. 10. Photodecomposition and subsequent chemical degradation of dacarbazine (2).

i.v. administration of dacarbazine to patients was 35 min (Loo et al., 1968). Measurement of plasma concentrations of dacarbazine using a HPLC method yielded a biphasic elimination profile with a terminal half-life of 41.4 min (Breithaupt et al., 1982). In this study, the plasma decay of metabolically generated AIC was monoexponential with a half-life of 43–116 min. The urinary recovery was 46–52% for dacarbazine and 9–18% for AIC. These pharmacokinetic data are in broad agreement with those obtained earlier with inferior analytical methods (Householder and Loo, 1969, 1971; Loo et al., 1968; Skibba et al., 1969a,b).

Dacarbazine is photolabile and reverts in light to its synthetic precursor 5-diazoimidazole-4-carboxamide (40, Fig. 10) (Horton and Stevens, 1981). This diazo compound and its decomposition products, 2-azahypoxanthine (41) and 4-carbamoylimidazolium-5-olate (42) have not been found as metabolites of dacarbazine in vivo, although the former has been implicated in the antibacterial action of the parent drug (Saunders and Schultz, 1972).

5. METABOLISM OF THE IMIDAZOTETRAZINONES

The metabolic fate of the imidazotetrazinones remains to be elucidated fully. Unlike the open chain dacarbazine which does not decompose under simulated physiological conditions, the cyclic mitozolomide (3) undergoes rapid hydrolytic ring-opening to give 5-[3-(2-chloroethyl)triazen-1-yl]imidazole-4-carboxamide (MCTIC: 44, Fig. 11) (Stevens et al., 1984) which may well be the ultimate antineoplastic metabolite of the parent drug (Horgan and Tisdale, 1984). MCTIC is an exceedingly short-lived compound with a $t_{1/2}$ of only 5.5 min at pH 7.5 (Horgan, 1985). Interestingly, this is the same compound as would be speculated to be formed by metabolic oxidative dealkylation of 5-[3,3-bis(2-chloroethyl)triazen-1-yl]imidazole-4-carboxamide (45), a dialkyltriazene with activity against tumors in the brain (Shealy et al., 1968; Levin et al., 1975). The great reactivity of 3-(2-chloroethyl)triazenes (46, Fig. 12) is thought to be due to their facile cyclization to 1,2,3-triazolines (48) or 1,2,3-triazolium salts (49) (Abraham et al., 1969; Lown and Singh, 1981). There is evidence to suggest that metabolism is an important determinant of the rate of deactivation of mitozolomide. Workman and Lee (1984) have shown that pretreatment of mice with phenobarbital reduces the activity of mitozolomide against the KHT sarcoma. In accordance with this finding, the area of the plasma-concentration-of-mitozolomide vs. time curve, determined in mice which had been pretreated with phenobarbital, was also significantly smaller than that in control mice (Brindley et al., 1986).

Hydrolytic ring-opening of temozolomide (4), the methyl analog of mitozolomide, should yield MTIC (38, Fig. 13). In order to test the hypotheses that temozolomide degrades to MTIC in physiological solutions and that MTIC mediates, or contributes to, the cytotoxicity of temozolo-

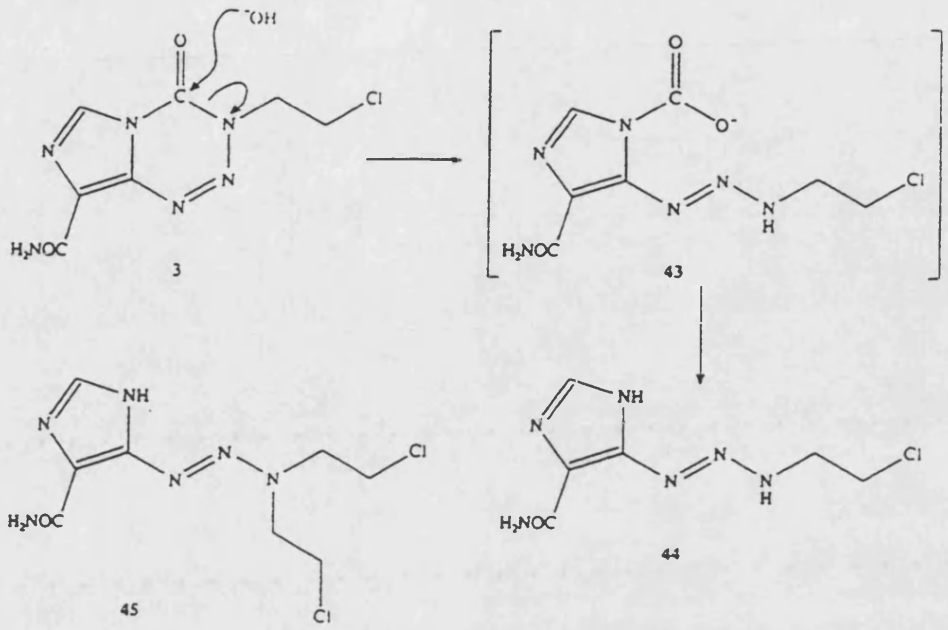

FIG. 11. Non-enzymic hydrolytic ring-opening of mitozolomide (3) to the active metabonate MCTIC (44).

mide, the drug was incubated with TLX5 cells. MTIC was detected by HPLC within 1 hr in the presence or absence of murine liver microsomes (Tsang et al., 1991). In contrast, when dacarbazine was incubated under the same conditions, MTIC was found only when microsomes were present. MTIC is also present in the urine of mice that have received temozolomide (Tsang et al., 1991). When temozolomide was incubated with preparation of murine liver, the drug was not metabolized at a measurable rate except for its chemical degradation to MTIC (Tsang et al., 1990). Contrastingly, the analogous 3-methylbenzo-1,2,3-triazin-4-one (52) was metabolically demethy-

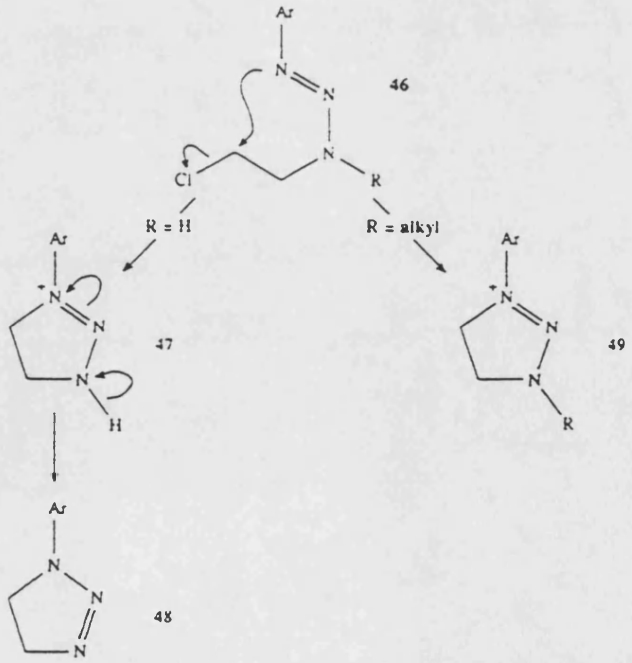

FIG. 12. Mechanism of cyclization degradation of *N*-(2-chloroethyl)triazenes.

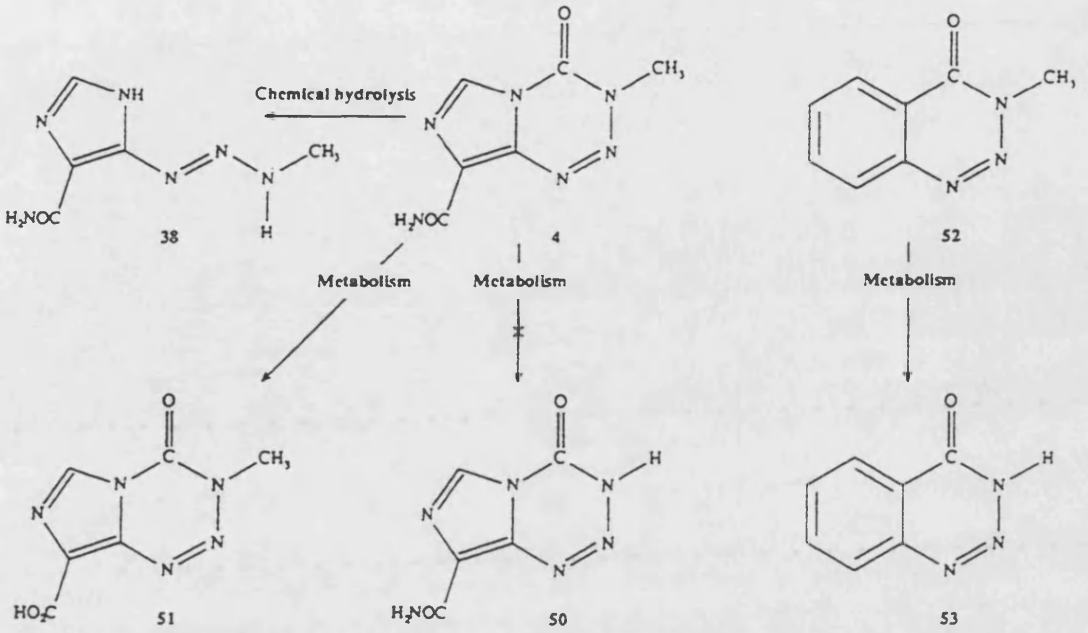

FIG. 13. Metabolic fate of temozolomide (4) in comparison with that of its non-antineoplastic analogue (52).

lated to give the benzotriazinone (53, Fig. 13). The major route of excretion of temozolomide in mice is via the kidneys. An acidic metabolite of temozolomide, probably a conjugate, was found in the urine of mice but its identity has not yet been established unambiguously. Another metabolite, the corresponding 8-carboxylic acid (51), has been found in the urine of human patients but not of mice. Unlike the unknown conjugate, this carboxylic acid was cytotoxic towards TLX5 lymphoma cells in vitro (Tsang et al., 1990).

6. THE ROLE OF METABOLISM IN THE MECHANISM OF ANTITUMOR ACTIVITY OF TRIAZENES

Many crucial mechanistic details that link the metabolism of triazenes with their antineoplastic activity remain obscure. Answers to the following three questions appear to be pivotal in the elucidation of this link:

(1) How can the aggressively reactive monomethyltriazene generated by metabolism of a dimethyltriazene cause selective cytotoxicity?

(2) Is the metabolic route which leads to the antineoplastic species identical with that which generates the carcinogenic metabolite?

(3) Are differences in the metabolism between dimethyltriazenes and other dialkyltriazenes responsible for the marked difference in antineoplastic activity observed in some murine tumors (Connors et al., 1976; Gescher et al., 1981)?

Even though the evidence for the involvement of metabolism in the mechanism of antitumor activity of dimethyltriazenes is overwhelming, the possibility that some dimethyltriazenes are themselves cytotoxic, under certain circumstances, without metabolic activation cannot be dismissed. Dacarbazine has been used in local therapy in patients with advanced malignant melanoma and soft tissue sarcoma of the extremities and regression of tumors has been observed (Aigner et al., 1983). Furthermore, 1-(4-carboxyphenyl)-3,3-dimethyltriazene (1; R 4-CO_2H, Fig. 1) did not undergo marked oxidative demethylation in vitro (Sava et al., 1982), yet it possessed antimetastatic properties in mice bearing the Lewis lung carcinoma (Giraldi et al., 1981). This drug also increased the survival of mice bearing the TLX5 lymphoma or the P388 leukemia without exerting marked cytotoxicity (Sava et al., 1982).

Some doubt has been cast on the contention that an electrophile as indiscriminate and non-selective as a monomethyltriazene could account for the antineoplastic activity of dimethyltriazenes (Gescher et al., 1981). This doubt was based on experiments in which the cytotoxicity of a monomethyltriazene was tested in a bioassay against a mouse TLX5 lymphoma cell line which had acquired resistance to dimethyltriazenes in vivo. The monomethyltriazene was found to be non-selectively toxic to both the sensitive and resistant lines, whereas incubation of the dimethyltriazene with a liver homogenate produced metabolites which were more toxic to the sensitive than to the resistant lymphoma cells (Gescher et al., 1981). However, cytotoxic metabolites of dimethyltriazenes, which are more selective than the monomethyltriazenes, have yet to be isolated or characterized. *N*-(Hydroxymethyl)triazenes were found to be equally as cytotoxic as their decomposition products, the monomethyltriazenes (Vaughan et al., 1984; Kohlsmith et al., 1984). Consequently, it has been suggested by some workers that the *N*-(hydroxymethyl) compounds may be the ultimate or proximate cytotoxic metabolites of dimethyltriazenes (Vaughan et al., 1984). The third question posed above has been addressed in a recent comparison of the antitumor activities and toxicities of 1-(4-acetylphenyl)-3,3-dimethyltriazene (20, Fig. 6) and 1-(4-acetylphenyl)3,3-diethyltriazene (24, Fig. 6) (Farina et al., 1986). The diethyltriazene was inactive against three murine tumors which were highly sensitive to the dimethyltriazene. However, the diethyltriazene was much more toxic to the host than was the dimethyl analogue. Both compounds were rapidly metabolized in vivo and in vitro and the monoalkyltriazenes (21 and 25, respectively) and 4-aminoacetophenone (22) were identified as metabolites of the parent compounds. However, the extent to which the drugs were *N*-dealkylated differed markedly. The amount of monoethyltriazene generated was only one third of the amount of monomethyl derivative found (Farina et al., 1986). This result suggests that the difference in activities between different dialkyltriazenes may indeed be due to differences in metabolism. A QSAR study has shown good correlation between antitumor activity and the presence of electron-donating substituents on the benzene ring of 3,3-dimethyl-1-(substituted)phenyltriazenes (Hatheway et al., 1978); this same chemical parameter also correlates with the extent of metabolic activation by hydroxylation at *N*-CH_3 by cytochromes P450 (Venger et al., 1979; Shusterman et al., 1989a,b). However, based upon studies involving induction of a cytochromes P450 by pretreatment with phenobarbital, Sava et al. (1988) raised some doubt about the role of metabolic activation in the antitumor activities and antimetastatic activities of 1-aryl-3,3-dimethyltriazenes against the Lewis lung carcinoma in the mouse. There has been one suggestion that arenediazonium metabonates of 1-aryl-3,3-dialkyltriazenes are responsible for their antitumor activity (Nifontov et al., 1984) but this conclusion is at variance with the vast majority of the literature.

On the basis of a preliminary pharmacokinetic study of dacarbazine and its metabolite MTIC in rodents and in human patients, Rutty (1983) suggested that the relative lack of antitumor activity of dacarbazine in man compared to rodents may be related to differences in the rate of hydroxylation at the *N*-methyl group; this hydroxylation is slow in man and fast in mice. It is, however, interesting to note that murine Sarcoma 180 cells themselves are capable of metabolizing dacarbazine (Mizuno and Humphrey, 1972).

Temozolomide is hydrolyzed chemically to its active metabonate MTIC, whereas this reactive electrophile is formed only after oxidative metabolism of dacarbazine. Thus, temozolomide may provide a pharmacokinetic advantage over dacarbazine in the clinical situation in the generation of the cytotoxic monomethyltriazene. A large number of analogues of mitozolomide and temozolomide have been synthesized with the aim of clarifying which chemical features of the imidazotetrazinone are essential for expression of their cytotoxic and antineoplastic properties. In analogues with a carboxamide at position 8, an NH appears to be required for maximal cytotoxicity in vitro. The dimethylcarboxamide 5 (Fig. 1) is markedly less cytotoxic in vitro than is mitozolomide (3) or the monomethylcarboxamide 6 (Horspool et al., 1989); nevertheless, the antitumor activity of the three agents in mice bearing the TLX5 lymphoma was very similar (Hickman et al., 1985; Lunt et al., 1987). The chemical stabilities of the three compounds are also similar. Incubation of 5 with marine hepatic microsomes led to increased cytotoxicity and to formation of the monomethyl analogue 6 (Horspool et al., 1989), suggesting that the latter causes or contributes to the antitumor effect of 5 in vivo. Interestingly, it has been demonstrated that $>89\%$ of the dose (10 mg kg^{-1}) of the administered dimethylcarboxamide is metabolized via the monomethylcarboxamide in the mouse.

7. THE ROLE OF METABOLISM IN THE MECHANISMS OF TOXICITY, MUTAGENICITY AND CARCINOGENICITY OF TRIAZENES

1-Aryl-3-methyltriazenes are direct-acting mutagens in the Ames assay (Malaveilie et al., 1982). The mutagenic activities of 1-aryl-3,3-dimethyltriazenes in *Drosophila melanogaster* have been reported to correlate with parameters on nucleophilicity which also correlate well with the extent of oxidative metabolic demethylation by cytochromes P450 (Vogel, 1989). Inhibition of cytochromes P450 causes a decrease in mutagenicity of 1-(2,4,6-trichlorophenyl)-3,3-dimethyltriazene (18) in this species, whereas inhibition of monoamine oxidase and of flavin monooxygenases caused increases in mutagenicity, suggesting again that metabolism by cytochromes P450 is required for mutagenic activity of this aryldimethyltriazene (Zijlstra and Vogel, 1988). In contrast, analogous studies with enzyme inhibitors indicated that the metabolism of dacarbazine (2) to the mutagenic derivative was mediated by flavin monooxygenases. It has been demonstrated that, for a wide range of aryldialkyltriazenes and heteroaryldimethyltriazenes (including dacarbazine), mutagenicity was manifest towards *Salmonella* in the Ames test after incubation with a rodent S9 microsomal preparation. In these QSAR studies, mutagenicity was found to correlate with the hydrophobicity (expressed as log P) and with parameters of electron density: extent of metabolism by cytochromes P450 also correlates with these parameters in a similar way (Venger et al., 1979; Hopfinger and Potenzone, 1982; Shusterman et al., 1989a,b). A strong relationship between toxicity to rats (expressed as LD_{50}) and these parameters has also been determined (Hansch et al., 1978).

Examples of benzenediazonium compounds, which are putative chemical breakdown products and metabolites of 3,3-dimethyl- or 3-methyl-triazenes, have been noted to be moderately carcinogenic in mice (Toth et al., 1989) and mutagens (Malaveille et al., 1982).

Kohn et al. (1988) have reported that the urine of patients who bave received dacarbazine (2) contains metabolites that are mutagenic to *Escherichia coli*.

8. CONCLUSIONS

It has been shown in very many studies that the principal metabolic process undergone by 1-aryl-3,3-dimethyltriazenes is oxidative hydroxylation at N-methyl group, with subsequent breakdown to the corresponding monomethyltriazene. It is also abundantly clear that this is a necessary metabolic step for the expression of beneficial antineoplastic activity of such triazenes, including the clinically used drug dacarbazine. Unfortunately, the molecular parameters which predispose these open-chain triazenes to metabolic activation as antitumor agents are effectively the same as those correlating with activation as mutagens, carcinogens and general toxins: oxidative metabolic activiation also requires that patients have competent hepatic function. Hence, one can only be pessimistic about the prospects of design of new dimethyltriazenes for therapeutic applications. Generation of the reactive and cytotoxic monomethyltriazenes non-enzymically from pro-drugs such as temozolomide may well constitute the way forward in the design of new compounds of this class for the effective treatment of cancers.

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PUBLICATION 65

Identification of 3'-Methoxy-4'-nitroflavone as a Pure Arylhydrocarbon (Ah) Receptor Antagonist and Evidence for more than One Form of the Nuclear Receptor in Human MCF-7 Human Breast Cancer Cells

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Identification of 3′-Methoxy-4′-nitroflavone as a Pure Aryl Hydrocarbon (Ah) Receptor Antagonist and Evidence for More Than One Form of the Nuclear Ah Receptor in MCF-7 Human Breast Cancer Cells[1]

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The competitive binding of 3′-methoxy-4′-nitro, 4′-amino-3′-methoxy, 4′-methoxy-3′-nitro, and 3′-amino-4′-methoxyflavone (compounds 1 to 4, respectively) to the rat cytosolic aryl hydrocarbon (Ah) receptor gave IC_{50} values of 2.27, 86.1, 872, and 19.4 nM. Flavones 3 and 4 were characterized as Ah receptor agonists in MCF-7 human breast cancer cells and induced CYP1A1 gene expression, whereas the 3-methoxy-substituted flavones (1 and 2) were inactive. All four compounds inhibited induction of ethoxyresorufin *O*-deethylase (EROD) activity by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in MCF-7 cells; moreover, *in vitro* studies with TCDD-induced rat liver microsomes showed that flavones 1 to 4 inhibited EROD activity in the presence or absence of NADPH. In MCF-7 cells cotreated with flavones 1 or 2 (0.01 to 10 μM) plus TCDD or [^{3}H]TCDD, there was a concentration-dependent inhibition of TCDD-induced CYP1A1 mRNA levels and formation of radiolabeled nuclear Ah receptor complex. Velocity sedimentation analysis of nuclear extracts from MCF-7 cells treated with [^{3}H]TCDD plus 1 or 10 μM concentrations of flavones 1 and 2 showed that an early eluting specifically bound nuclear Ah receptor complex was present. However, under these same conditions the flavones inhibited TCDD-induced CYP1A1 gene expression. The apparent molecular mass of this nuclear complex was 190 kDa as determined by cross-linking to a ^{32}P-labeled bromodeoxyuridine-substituted consensus dioxin-responsive element. Similar cross-linking results were obtained using the nuclear extract from MCF-7 cells treated with [^{3}H]TCDD alone. The results of this study suggest that there are at least two forms of the nuclear Ah receptor complex in MCF-7 cells; the major transcriptionally active form binds [^{3}H]TCDD and flavones 1 or 2 inhibit nuclear uptake of this receptor complex. The other form of the nuclear Ah receptor complex appears to be transcriptionally inactive and ligand binding with [^{3}H]TCDD is not competitively inhibited by flavones 1 and 2.

Key Words: cytosolic receptors, transport, intracellular signaling.

Bioflavanoids and related synthetic analogs exhibit a broad spectrum of biological activity. Naturally occurring plant flavanoids such as naringenin (flavanone), apigenin (flavone), and genistein (isoflavone) which are substituted with hydroxyl groups at the 4′ and 3, 5, or 7 positions are weak estrogens which bind to the estrogen receptor and elicit estrogen-induced responses (1–4). Other studies have reported that naturally occurring polyhydroxylated flavanoids, chalcones, and structurally related synthetic analogs exhibit antimutagenic and anticarcinogenic activities (5–12) and inhibit activities of a number of enzymes including protein kinases (13–16), porcine-5-lipoxygenase (17), ornithine decarboxylase (11), glutathione reductase (18), P450 isozymes (19), and HIV proteinase (20). Bioflavanoids can also enhance some P450-dependent activities, inhibit or enhance carcinogen-induced tumors or DNA binding (10, 12, 21–25) and inhibit human platelet aggregation (19). The flavonoid-mediated effects are dependent on numerous factors including the structure of the compound, the target organ or cell, and the response.

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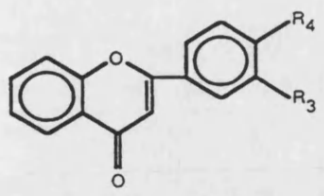

	R_4	R_3
1	NO_2	OCH_3
2	NH_2	OCH_3
3	OCH_3	NO_2
4	OCH_3	NH_2

FIG. 1. Structures of 3′-methoxy-4′-nitroflavone (1), 4′-amino-3′-methoxyflavone (2), 4′-methoxy-3′-nitroflavone (3), and 3′-amino-4′-methoxyflavone (4).

The synthetic flavone, 5,6-benzoflavone (β-naphthoflavone), inhibited carcinogen-induced tumor formation in the mouse skin model and this activity was related, in part, to inhibition of P450-dependent metabolic activation of carcinogens (9–12). Interestingly, β-naphthoflavone binds to the aryl hydrocarbon (Ah)[4] receptor and is often used as a prototypical inducer of Ah receptor-mediated CYP1A1 and CYP1A2 gene expression (19, 26, 27). 7,8-Benzoflavone (α-naphthoflavone, αNF) binds with moderate affinity to the Ah receptor and has been extensively characterized as a partial Ah receptor antagonist (28–32). In a recent study, it was shown that in various cancer cell lines, at concentrations $\leq 10^{-6}$ M, αNF inhibited 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced CYP1A1 gene expression and this was paralleled by decreased formation of the nuclear Ah receptor complex (32). In contrast, αNF was an Ah receptor agonist at a concentration of 10^{-5} M (33).

Current studies in this laboratory have been investigating the modulatory effects of various, 3′,4′-substituted flavones on Ah receptor-mediated signal transduction pathways and this report describes the activity and specificity of the 3′-methoxy-4′-nitro, 4′-amino-3′-methoxy-, 4′-methoxy-3′-nitro-, and 3′-amino-4′-methoxyflavones (Fig. 1) as Ah receptor agonists and antagonists in the MCF-7 human breast cancer cell line. All four flavones rapidly inactivate CYP1A1-dependent activity and results from *in vitro* studies suggest that this is due to metabolically mediated suicide inactivation of CYP1A1 protein. In contrast, the Ah receptor binding affinities and Ah receptor agonist/antagonist activities of flavones 1 to 4 are remarkably dependent on structure. Both 3′-methoxy-4′-nitro- and 4′-amino-3′-methoxyflavone inhibit TCDD-induced responses in MCF-7 cells and the results obtained for the former compound indicate that it is a "pure" Ah receptor antagonist.

MATERIALS AND METHODS

Chemicals and biochemicals. The 3′,4′-substituted flavones were synthesized as described (16). TCDD, [^{3}H]TCDD (37 Ci/mmol), ethoxyresorufin, and 2,3,7,8-tetrachlorodibenzofuran (TCDF) were synthesized in this laboratory to >98% purity. MCF-7 human breast cancer cells were originally obtained from American Type Culture Collection (ATCC, Rockville, MD). All other chemicals and biochemicals were of the highest purity available from commercial sources.

Preparation of rat hepatic cytosol. Rat hepatic cytosol was prepared essentially as described earlier from male Long-Evans rats (34). Livers were perfused *in situ* with ice-cold HEGD buffer [25 mM Hepes, 1.5 mM EDTA, 1.0 mM dithiothreitol, 10% glycerol (v/v), pH 7.8] and homogenized in HEGD (5 ml/g tissue) using a Brinkman homogenizer. The homogenates were centrifuged at 10,000*g* for 20 min (2°C); the resulting supernatant was centrifuged at 105,000*g* for 1 h (2°C), removed with a Pasteur pipette, and stored at −80°C. Protein concentrations were determined as described earlier (35).

Hydroxyapatite (HAP) assay. The IC_{50} and K_i values for competitive receptor binding affinities were determined using frozen rat hepatic cytosol (2 mg protein/ml) and the HAP procedure essentially as described using 1 nM [^{3}H]TCDD as the radioligand (34). Different concentrations of 3′,4′-substituted flavones were used to determine displacement curves. The IC_{50} values were defined as the concentrations required to displace 50% of the [^{3}H]TCDD and were determined from a probit plot of the percentage of [^{3}H]TCDD bound versus log concentrations of the ligand. K_i values were determined by the methods of Cheng and Prusoff (36) in which the IC_{50} value for unlabeled TCDD was 1.28 nM (34).

Cell cytosol preparation, in vitro Ah receptor transformation, and gel retardation assay. Cytosol from rat liver was prepared as described (34) and incubated with different concentrations of the test compounds at 20°C for 2 h. Ligand-induced Ah receptor transformation was determined by gel retardation analysis (34). Complementary strands of synthetic oligonucleotides containing the DRE sequence 5′-GATCTGGCTCTTCTCACGCAACTCCG-3′ were synthesized, purified by polyacrylamide gel electrophoresis, and annealed (37). Nuclear extracts or transformed cytosol from MCF-7 cells were used for the gel retardation analysis as previously described (37) and the specifically bound retarded DRE-Ah receptor was quantitated using a Betagen Betascope 630 blot analyzer or by autoradiography.

Inhibition of TCDD-induced rat liver microsomal EROD activity. Rat liver microsomes were isolated from TCDD (5 μg/kg)-treated male Long-Evans rat liver (38). Test compounds were incubated with TCDD-induced rat liver microsomes, bovine serum albumin, magnesium sulfate, NADPH, and NADH at 37°C for 2 or 5 min and EROD activity was determined fluorimetrically (39).

Cell growth and EROD induction assay. MCF-7 cells were routinely grown in DME/F12 with 2.2 mg/ml sodium bicarbonate, 5% fetal calf serum, and 10 nM antibiotic–antimycotic solution (Sigma Chemical Co., St. Louis, MO). For the EROD induction assay, the cells were seeded into 60-mm Petri dishes. TCDD and the substituted flavones dissolved in DMSO (0.1%) were added to the culture dishes when the cells reached about 70% confluence. Cells were harvested 24 h after the chemical treatment and EROD activity was determined by the method of Pohl and Fouts as previously described (39).

Preparation of nuclear extracts. Near confluent MCF-7 cells were trypsinized and washed with the culture medium. Harvested cells were resuspended in 10 ml culture medium in 25-cm^2 culture flasks (about 5×10^6 cells/ml). For the Ah receptor binding assay, [^{3}H]TCDD (10 nM) with or without different concentrations of flavones 1 to 4 was added to the cell culture flasks. The cells were incubated by gentle shaking for 2 h at 37°C. After incubation, the suspended cells were harvested and washed with 30 ml HEGD buffer. The washed cell pellet was resuspended in 3 ml of HED buffer (same as HEGD buffer without glycerol) and incubated at 4°C for 10 min. Repelleted cells were transferred to a 2-ml homogenizing tube, 1 ml of HEGD buffer was added, and the cell suspension was homogenized. The homogenate was transferred and washed with HEGD buffer and centrifuged; the resulting nuclear pellet was then resuspended in extraction buffer (HEGD + 0.5

[4] Abbreviations used: Ah, aryl hydrocarbon; αNF, α-napthoflavone; DRE, dioxin-responsive element; EROD, ethoxyresorufin *O*-deethylase; MCDF, 6-methyl-1,3,8-trichlorodibenzofuran; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDF, tetrachlorodibenzofuran; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

M KCl) and incubated at 4°C for 1 h. After centrifugation, the supernatant extracts which contained the nuclear Ah receptor protein were aliquotted and stored at −70°C until used. Protein concentrations were determined according to Bradford (35).

Sucrose density gradient analysis of radiolabeled nuclear Ah receptor. Aliquots (200 μl) of nuclear extract were layered onto linear sucrose gradient (5 to 25%) prepared in HEGD buffer. Gradients were centrifuged at 4°C for 2.5 h at 400,000*g*. After centrifugation, 30 fractions were collected from each gradient and radioactivity in each fraction was determined by liquid scintillation counting to give the total binding. Nonspecific binding was determined in cells treated with radiolabeled TCDD plus 200-fold excess of unlabeled TCDF. Specific binding was determined from the difference between the total and nonspecific binding as described (34, 40).

cDNA sources and CYP1A1 mRNA analysis. The murine CYP1A1 (1.2 kb) cDNA probe was obtained from the American Type Culture Collection. The plasmid pGMB1.1 containing the mouse β-tubulin cDNA was a gift from Dr. Don Cleveland (Johns Hopkins University). Digestion of the plasmid yielded a 1.3-kb fragment which was used to detect β-tubulin mRNA. RNA from the cells treated with TCDD or substituted flavones for 24 h was isolated, electrophoresed, transferred to a nylon membrane, and probed by autoradiography or using a Betagen Betascope 603 imaging system as previously described (30, 31, 34).

Preparation of BrdU-substituted DNA. For cross-linking studies, 10 pmol of the synthetic oligonucleotide, 5'-GATCTCCGGTCCCTTCTCACGCAACGCCTGGGC-3', was annealed to a 10 pmol of a 7-bp complementary primer, 5'-GCCCAGG-3'. The annealed template was end-filled with the Klenow fragment of DNA polymerase in the presence of 0.1 μM dGTP, dATP, BrdU, and 1 μM [^{32}P]dCTP as described (41) and was designated as the BrdU-substituted DRE oligonucleotide.

Ultraviolet cross-linking. Nuclear extracts (10 μg) were incubated at 20°C with 400 ng of poly[d(I-C)] in HEGD buffer followed by the addition of a BrdU-substituted ^{32}P-labeled DRE and incubated for 15 min at 20°C. The gel mobility shift assay products were uv irradiated directly on a FOTODYNE uv transilluminator, >205 nm for 20 min at 20°C. Samples were then mixed with 20 μl of an SDS-loading buffer, heated to 95°C for 5 min, and then subjected to electrophoresis on 10% SDS–polyacrylamide gels. Ligand:Ah receptor:BrdU-DRE complexes were resolved by autoradiography of the dried gel. The molecular weight of uv-cross-linked nuclear ligand:Ah receptor:BrdU-DRE complexes were determined by [^{14}C]methylated protein standards obtained from Amersham Corp. (Arlington Heights, IL).

Statistical analysis. The statistical differences between different treatment groups were determined by ANOVA and Student *t* test and the levels of probability are noted ($P < 0.05$ or $P < 0.01$). The data are expressed as means ± standard errors (SE) or deviations (SD) for at least three determinations for each experimental point.

RESULTS

Using rat hepatic cytosol, the concentration-dependent displacement of [^{3}H]TCDD by the substituted flavones (1 to 4) was determined. All the compounds competitively displaced [^{3}H]TCDD from the receptor and the IC_{50} values (means ± SE) were 2.27 ± 0.61, 86.1 ± 6.3, 872 ± 362, and 19.4 ± 12 nM for flavones 1 to 4, respectively (Table I). Thus, 3'-methoxy-4'-nitroflavone exhibits competitive Ah receptor binding affinity which is not significantly different from unlabeled TCDD (IC_{50} = 1.78 nM) run in the same assay system. The corresponding K_i values calculated for compounds 1 to 4 were 1.46 ± 0.39, 55.2 ± 4.0, 559 ± 232, and 12.4 ± 7.7 nM, respectively. Transformation of rat hepatic cytosolic Ah receptor by flavones was also investigated using gel retardation assays and DRE binding; compared to the DRE binding observed for 1 nM TCDD, 10 μM concentrations of flavones 1 to 3 gave transformed DRE complexes which were <6% of that observed for 1 nM TCDD. In contrast, 10 μM 3'-amino-4'-methoxyflavone significantly transformed rat hepatic cytosolic Ah receptor (Table I).

TABLE I

Competitive Binding of Flavones 1 to 4 to Rat Hepatic Cytosol Ah Receptor and Their Activity to Induce Transformation of the Rat Cytosolic Ah Receptor[a]

Compound	IC_{50} (nM)[a]	K_i (nM)[a]	DRE binding[b]
3'-Methoxy-4'-nitroflavone	2.27 ± 0.61	1.46 ± 0.39	ND
4'-Amino-3'-methoxyflavone	86.1 ± 6.3	55.2 ± 4.0	5.3
4'-Methoxy-3'-nitroflavone	872 ± 362	559 ± 232	1.2
3'-Amino-4'-methoxyflavone	19.4 ± 12	12.4 ± 7.7	92

[a] The IC_{50} values were determined in competitive binding studies using [^{3}H]TCDD as the radioligand and different concentrations of unlabeled competitor as described under Materials and Methods; K_i values were calculated from the IC_{50} values. The results are expressed as means ± SE for four separate determinations.

[b] DRE binding was also determined using rat hepatic cytosol as described (34); the results are expressed as DRE binding observed for 10 μM concentrations of the flavones 1 to 4 as a percentage of that observed using 1 nM TCDD in the transformation assay.

The results in Fig. 2A illustrate the concentration-dependent induction (0.01 to 10 μM) of EROD activity by flavones 1 to 4 in MCF-7 cells. No significant induction of EROD activity was observed for these compounds (1 to 4) after incubation with MCF-7 cells for 24 h. In contrast, the EROD activity observed for 1 nM TCDD was 110 pmol/min/mg. The interactive effects of the substituted flavones and TCDD were also investigated in MCF-7 cells treated with 1 nM TCDD plus different concentrations of the flavones (0.01 to 10 μM) for 24 h (Fig. 2B). No significant inhibition of induced EROD activity was observed in cells cotreated with 1 nM TCDD plus 10 nM concentration of the flavones; some compound-dependent inhibition was observed at a 100 nM concentration, whereas total inhibition of TCDD-induced EROD activity was observed in cells cotreated with 1 nM TCDD plus 1 μM flavones 1 to 4. Inhibition of EROD activity by flavones 1 to 4 was further investigated by incubating these compounds with TCDD-induced rat liver microsomes. The results (Fig. 3) demonstrated that flavones 1 to 4 rapidly inactivated CYP1A1-dependent EROD activity. This response was observed in the presence of NADPH; however, significant inhibition was also noted when this cofactor was not included in the incubation mixture (data not shown).

MCF-7 cells were treated with 1 nM TCDD (alone), 1 μM concentrations of the synthetic flavones 1 to 4, and

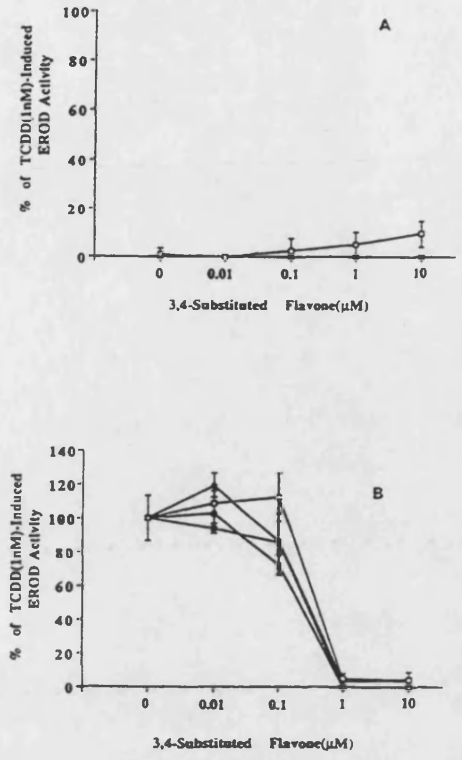

FIG. 2. Effects of flavones on induction of EROD activity (A) and inhibition of TCDD-induced EROD activity (B) in MCF-7 human breast cancer cells. The concentration-dependent induction of EROD activity by flavones 1 to 4 showed that only 3'-amino-4'-methoxyflavone (□) caused a minimal induction response at the highest concentration (10 μM) (A) after 24 h. In the cotreatment study (B), MCF-7 cells were cotreated with 1 nM TCDD and 0.01 to 10 μM concentrations of flavones 1 (●), 2 (○), 3 (■), and 4 (□) for 24 h; EROD activity was determined fluorimetrically as described and the results are expressed as means ± SD for at least three determinations for each treatment group. EROD activity in cells treated with 1 nM TCDD alone was 110 pmol/min/mg.

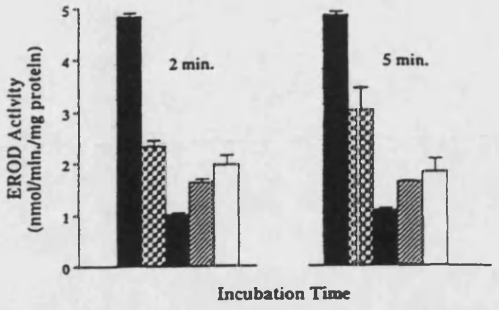

FIG. 3. Effects of flavones 1 to 4 on EROD activity of hepatic microsomes from rats treated with TCDD (5 μg/kg). Rat liver microsomes and reduced nucleotide cofactors were incubated with 1 μM concentrations of 4'-methoxy-3'-nitro- (▨), 4'-amino-3'-methoxy- (■), 3'-methoxy-4'-nitro- (▨), and 3'-amino-4'-methoxy-flavone (□) for 2 (left) or 5 (right) min prior to determination of EROD activity. EROD was determined fluorimetrically in the control (■) and flavone-treated microsomes and there was a significant decrease ($P < 0.05$) in EROD activity in microsomes treated with flavones 1 to 4 after 2 and 5 min. Results are expressed as means ± SD for at least three determinations.

a combination of 1 nM TCDD plus 1 μM concentrations of the flavones and the induction of CYP1A1 mRNA levels were determined by Northern blot analysis. The results (Table II) showed that flavones 1 to 3 alone elicited no significant induction of CYP1A1 mRNA levels, whereas 1 μM 3'-amino-4'-methoxyflavone induced CYP1A1 mRNA levels comparable to that observed for 1 nM TCDD. In the cotreatment studies, both 3'-methoxy-4'-nitro- and 4'-amino-3'-methoxyflavone inhibited induction of CYP1A1 mRNA levels by TCDD. In contrast, the 4'-methoxy substituted flavones did not inhibit TCDD-induced CYP1A1 mRNA levels. The concentration-dependent inhibition of TCDD-induced CYP1A1 mRNA levels by 3'-methoxy-4'-nitro- and 4'-amino-3'-methoxyflavone is illustrated in Fig. 4. The former compound alone is inactive as an inducer at concentrations as high as 10 μM and the inhibitory effects in the cotreated cells were observed at 1 and 10 μM concentrations. Similar results were observed for 4'-amino-3'-methoxyflavone; however, the compound alone at the highest concentration (10 μM) induced small but detectable CYP1A1 mRNA levels as determined by Northern blot analysis using the Betagen Betascope 603 blot analyzer imaging system (data not shown). The induction was not evident in the autoradiograph (Fig. 4).

TABLE II
Effects of Flavones 1 to 4 in the Presence or Absence of TCDD on Induction of CYP1A1 Gene Expression in MCF-7 Cells[a]

Treatment	Conc. (nM)	CYP1A1 mRNA levels
DMSO	—	0.9 ± 1.1
TCDD	1	36.5 ± 5.9[b]
3'-Methoxy-4'-nitroflavone	1000	0.5 ± 0.6
4'-Amino-3'-methoxyflavone	1000	0.4 ± 0.5
4'-Methoxy-3'-nitroflavone	1000	2.8 ± 0.4
3'-Amino-4'-methoxyflavone	1000	35.9 ± 16.8[b]
3'-Methoxy-4'-nitroflavone + TCDD	1000 + 1	1.1 ± 0.7[c]
4'-Amino-3'-methoxyflavone + TCDD	1000 + 1	0.6 ± 0.8[c]
4'-Methoxy-3'-nitroflavone + TCDD	1000 + 1	40.7 ± 0.4[b]
3'-Amino-4'-methoxyflavone + TCDD	1000 + 1	35.0 ± 22.9[b]

[a] MCF-7 cells were treated with 1 nM TCDD, 1000 nM flavones 1 to 4 in the presence or absencce of 1 nM TCDD for 24 h; mRNA was isolated and quantitated by Northern blot analysis (relative to β-tubulin mRNA) as described under Materials and Methods. The results are expressed as means ± SD for at least three determinations for each data point.

[b] Significantly higher ($P < 0.05$) than in control cells.

[c] Significantly lower ($P < 0.05$) than in cells treated with TCDD alone.

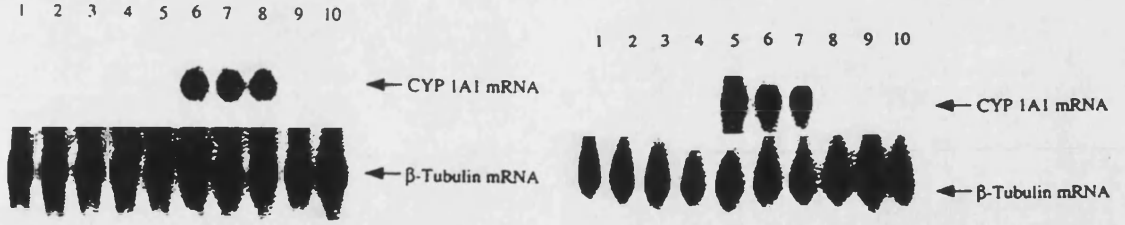

FIG. 4. Effects of 3′-methoxy-4′-nitroflavone (top) and 4′-amino-3′-methoxyflavone (bottom) as inducers of CYP1A1 mRNA levels in the presence or absence of 1 nM TCDD in MCF-7 cells. mRNA was extracted from MCF-7 cells 24 h after treatment with 0.01, 0.1, 1.0, and 10 μM 3′-methoxy-4′-nitroflavone, DMSO, 1 nM TCDD, 1 nM TCDD + 0.01, 0.1, 1.0, or 10 μM 3′-methoxy-4′-nitroflavone (lanes 1 through 10, respectively) (left); lanes 1 through 10 in right hand gel represent mRNA from cells treated with 0.01, 0.1, 1.0, and 10 μM 3′-methoxy-4′-aminoflavone, 1 nM TCDD, 1 nM TCDD plus 0.01, 0.1, 1.0, or 10 μM 3′-methoxy-4′-aminoflavone, and DMSO, respectively. mRNA was extracted and Northern blot analysis was carried out as described under Materials and Methods.

The effects of 3′-methoxy-4′-nitro- and 4′-amino-3′-methoxyflavone on formation of the nuclear Ah receptor complex were also determined in MCF-7 cells treated with 10 nM [^{3}H]TCDD and different concentrations of the two flavones. Both compounds inhibited formation of the nuclear Ah receptor complex (Fig. 5) in MCF-7 cells. However, an early eluting specifically bound peak which constituted approximately 10% of the total specifically bound nuclear Ah receptor complex was not affected by the flavones.

Nuclear extracts from MCF-7 cells treated with 10 nM [^{3}H]TCDD in the presence or absence of 1 μM 3′-methoxy-4′-nitro- or 4′-amino-3′-methoxyflavone were incubated with a [^{32}P]labeled BrdU-substituted DRE, irradiated with ultraviolet light for 20 min, and analyzed by gel retardation assays. Specifically bound cross-linked retarded bands (see arrow) were observed at 190 kDa by SDS–PAGE for all nuclear extracts, indicating that the nuclear Ah receptor complex in cells treated with only [^{3}H]TCDD or [^{3}H]TCDD plus the Ah receptor antagonists formed a DNA binding complex with similar *M*r values and consistent with a nuclear heterodimer of the Ah receptor and Arnt proteins (42). In addition, a lower molecular weight Ah receptor:BrdU-DRE complex (~97 kDa) was observed in MCF-7 cells treated with 1 μM 3′methoxy-4′nitro- or 4′amino-3′-methoxyflavone plus TCDD that was not observed in cells treated with TCDD alone.

DISCUSSION

Previous studies have reported that several different structural classes of compounds including 6-methyl-1,3,8-trichlorodibenzofuran (MCDF) and related 6-substituted analogs and αNF inhibit diverse TCDD-induced responses *in vivo* and in mammalian cells in culture (30–33, 40, 43). Both MCDF and related compounds and αNF competitively bind to the Ah receptor and block formation of the nuclear Ah receptor complex in various mammalian cells in culture (30). However, both MCDF and αNF also exhibit Ah receptor agonist activity in mammalian cells at high concentrations (32, 33). Flavonoids exhibit a broad spectrum of activities and this report describes interactions of a series of structurally related 3,4-disubstituted compounds containing a methoxy group at one and a nitro or amino substituent at the other position (Fig. 1) with the Ah receptor. These compounds were selected for this study following an extensive prescreening of a series of synthetic 3′-, 4′-, and 3′,4′-substituted flavones (16) as competitive ligands for the Ah receptor (data not shown). These compounds were previously prepared and their activities as protein kinase C inhibitors were reported (16). The competitive IC_{50} values for binding of the substituted flavones (1 to 4) to the rat hepatic cytosolic Ah receptor are summarized in Table I. The results indicate that for these structurally related flavones, there were marked effects of substituent structure and position on Ah receptor binding affinities. For example, 3′-methoxy-4′-nitroflavone exhibited a competitive Ah receptor binding affinity 384-fold higher than the isomeric 4′-methoxy-3′-nitroflavone and the IC_{50} value for the former compound was not significantly different than that observed for unlabeled TCDD (IC_{50} = 1.78 nM) (34). A ≥fourfold difference in binding affinity was observed for the isomeric 4′-amino-3′-methoxy- and 3′-amino-4′-methoxyflavones; however, for these isomers, the 4′-methoxy-substituted isomer exhibited the higher binding affinity. These substituent structure-binding relationships have not previously been observed for any other structural class of Ah receptor ligands. In previous studies with substituted biphenyls, dibenzo-*p*-dioxins, and dibenzofurans (44–46), substituent electronegativity, lipophilicity, and size$_e$ were the major structural determinants which facilitated binding to the rat cytosolic Ah receptor; not surprisingly, the methoxy- and amino-substituted analogs exhibited low binding affinities, whereas nitro-substituted compounds exhibited slightly increased binding. Thus, the high competitive binding affinities of 3′-methoxy-4′-nitro- and 3′-amino-4′-methoxyflavone (Table I) were unexpected based on the results of previous studies. The

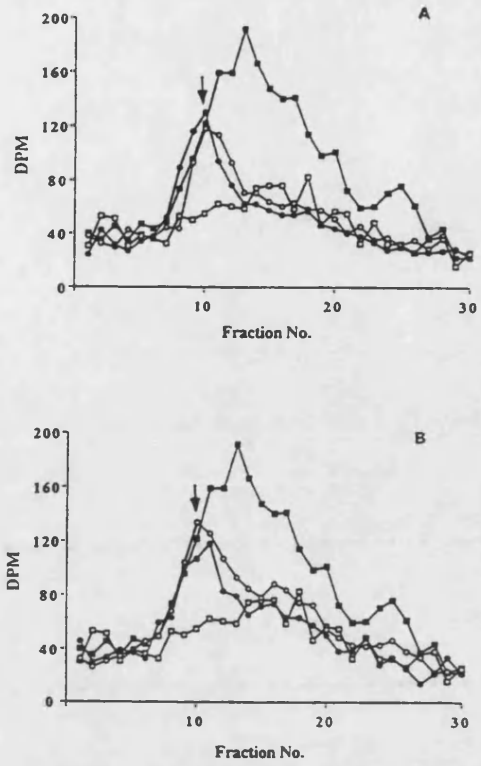

FIG. 5. Velocity sedimentation analysis of nuclear extracts from MCF-7 cells treated with 10 nM [^{3}H]TCDD in the absence or presence of 1 or 10 μM unlabeled 3'-methoxy-4'-nitroflavone (A) or 4'-amino-3'-methoxyflavone (B). MCF-7 cells were treated with [^{3}H]TCDD and different concentrations of the flavones. Nuclear extracts were obtained and analyzed by sucrose density gradient centrifugation as described under Materials and Methods section. Levels of specifically bound radiolabeled nuclear Ah receptor complex in cells treated with (A) 10 nM [^{3}H]TCDD (■), 10 nM [^{3}H]TCDD plus 1 μM 3'-methoxy-4'-nitroflavone (○), 10 nM [^{3}H]TCDD plus 10 μM 3'-methoxy-4'-nitroflavone (●), (B) 10 nM [^{3}H]TCDD plus 1 μM 4'-amino-3'-methoxyflavone (○), and 10 nM [^{3}H]TCDD plus 10 μM 4'-amino-3'-methoxyflavone (●) were 27.6 ± 1.48, 4.78 ± 0.46, 4.37 ± 1.48, 11.0 ± 1.44, and 6.40 ± 3.55 fmol/mg, respectively. The results are expressed and means ± SD for at least three separate experiments for each treatment group. Nonspecifically bound radioactivity was obtained from cells treated with 10 nM [^{3}H]TCDD and a 200-fold excess of unlabeled TCDF (□).

data obtained for flavones **1** to **4** further extend the structure-binding relationships for the Ah receptor in which compounds with highly variable aromatic ring and substituent structure bind to the Ah receptor. These data suggest that there may be more than one form of the Ah receptor or that the ligand-binding domain of the Ah receptor may contain multiple and/or possibly overlapping ligand-binding sites capable of interacting with structurally diverse compounds. Since the human Ah receptor gene has been cloned (47), mutational analysis of the ligand binding domain can be used in future studies to investigate the structural requirements for ligand interaction with the receptor protein binding site(s).

The activity of the 3',4'-substituted flavones **1** to **4** as inducers of CYP1A1 gene expression was determined in MCF-7 human breast cancer cells. The results illustrated in Fig. 2A indicate that the flavones do not significantly induce EROD activity in these cells at concentrations as high as 10 μM. Minimal or nondetectable induction of CYP1A1 mRNA levels was observed for flavones **1** to **3**, whereas 3'-amino-4'-methoxyflavone significantly induced CYP1A1 mRNA levels (Table II) and induced transformation of the rat cytosolic Ah receptor (Table I). The lack of correspondence between induced EROD activity versus CYP1A1 mRNA levels for flavone **4** was further investigated by cotreating MCF-7 cells for 24 h with 1 nM TCDD plus different concentrations of flavones **1** to **4** (Fig. 2B) or by adding these compounds directly to TCDD-induced microsomes (Fig. 3). The results demonstrate that all four compounds rapidly inactivate CYP1A1-dependent activity; however, the mechanism of this inhibitory response is unknown. These results indicate that the disparity between the induction of CYP1A1 mRNA levels by 3'-amino-4'-methoxyflavone (**4**) and the failure to observe induced EROD activity is due to inactivation of CYP1A1. Current studies are utilizing other substituted flavones to investigate the mechanism and specificity of their interaction with P450 isozymes.

The structure-induction (CYP1A1) relationships observed for flavones **1** to **4** did not parallel their structure-binding relationships (Table I) since only one compound (**4**) induced CYP1A1 gene expression, whereas 3'-methoxy-4'-nitroflavone (**1**), the congener with the highest receptor binding affinity (Table 1), was inactive as an inducer of CYP1A1 mRNA levels or EROD activity. The relatively high receptor binding affinities of flavones **1** to **3** for the Ah receptor coupled with their failure to induce CYP1A1 mRNA levels suggested that these compounds may be Ah receptor antagonists. Therefore, MCF-7 cells were cotreated with 1 nM TCDD plus flavones **1** to **4** (1 μM) and the results summarized in Table II show that only flavones **1** and **2** inhibit induction of CYP1A1 mRNA levels by TCDD. The inhibitory effects of these compounds was further investigated and the results illustrated in Fig. 4 show that at concentrations of flavones **1** to **2** which were $\geq$0.1 μM, significant inhibition of TCDD-induced CYP1A1 mRNA levels was observed. In this study, 4'-amino-3'-methoxyflavone induced minimal but detectable induction of CYP1A1 mRNA at the highest concentration (Fig. 4), whereas no induction response was observed for 3'-methoxy-4'-nitroflavone at a 10 μM concentration. Previous studies with the partial Ah receptor agonists, MCDF and αNF, showed that these compounds also inhibited TCDD-induced CYP1A1 mRNA levels and formation of the nuclear Ah receptor complexes (32, 33) at concentrations $\leq 10^{-6}$ M but exhibited agonist activity at higher concentrations. In contrast, flavones **1** or **2** at concentrations of 1 and 10

FIG. 6. Ultraviolet cross-linking of the nuclear ligand:Ah receptor complex to a BrdU-substituted DRE. Nuclear extracts (10 μg) from MCF-7 cells were incubated with ^{32}P-labeled BrdU-substituted (140,000 cpm, 0.5–1.0 ng) consensus DRE oligonucleotide as described under Materials and Methods. Samples were uv-irradiated for 30 min and subsequently analyzed by SDS–PAGE. Ligand:Ah receptor:DNA complexes were resolved by autoradiography. The arrows indicate the molecular weights based on the migration of radiolabeled standards. The cross-linked Ah receptor complexes were derived from nuclear extracts from MCF-7 cells treated with 10 nM TCDD (lane 1), 10 nM TCDD plus 1 μM flavone 1 (lane 2), and 10 nM TCDD plus 1 μM flavone 2 (lane 3), and 10 nM TCDD plus incubation with 100-fold excess unlabeled BrdU-substituted DRE (lane 4).

μM significantly inhibited induction of CYP1A1 mRNA levels by TCDD in MCF-7 cells (Fig. 4). Since 3′-methoxy-4′-nitroflavone exhibited no detectable Ah receptor agonist activity (Table II and Fig. 4), this compound is the first "pure" Ah receptor antagonist for MCF-7 cells which has hitherto been identified.

The results in Fig. 5 illustrate the velocity sedimentation profile of nuclear extracts from MCF-7 cells treated with 10 nM [^{3}H]TCDD in the presence or absence of flavones **1** or **2**. The specifically labeled nuclear Ah receptor complex sedimented as a broad specifically bound peak as determined using a 200-fold excess of unlabeled TCDF as a competitor (Fig. 5). In contrast, a portion of the specifically bound early eluting nuclear Ah receptor complex (see arrow) was observed in MCF-7 cells co-treated with [^{3}H]TCDD and flavones **1** or **2** (1 and 10 μM). The failure to inhibit formation of this specifically bound Ah receptor complex was consistently observed with both flavone antagonists. Previous studies with αNF or MCDF showed that these compounds also inhibited TCDD-induced CYP1A1 gene expression and formation of the nuclear Ah receptor complex (30–33). However, the formation of the early eluting Ah receptor complex was not detected in nuclear extracts. Incubation of nuclear extracts from MCF-7 cells treated with TCDD in the presence or absence of flavones **1** and **2** with ^{32}P-labeled BrdU-DRE followed by irradiation gave cross-linked complexes which were separated by electrophoresis (Fig. 6). The apparent molecular masses of the cross-linked complexes were 190 kDa and this is consistent with covalent interaction of both the Ah receptor and the Arnt proteins with the BrdU-DRE (42). The lower molecular weight cross-linked-monomer complexes were not detected in this assay. Thus, despite the different velocity sedimentation properties of the nuclear Ah receptor complexes (Fig. 5), the apparent molecular weights of the major cross-linked proteins were similar. The failure of flavones **1** and **2** to inhibit formation of the early eluting specifically bound nuclear Ah receptor complex suggests that [^{3}H]TCDD interacts with more than one binding site in the Ah receptor or that there is more than one form of the Ah receptor which exhibits differential competitive inhibition with flavones **1** and **2**. Results of studies in other laboratories also suggest that there may be more than one form of the Ah receptor (48–52). Moreover, since flavones **1** and **2** inhibit TCDD-induced CYP1A1 gene expression in MCF-7 cells, the early eluting specifically bound complex which forms in the nucleus must be inactive as a transcriptional enhancer for induction of CYP1A1 gene expression.

In summary, the results of this study demonstrate that 3′-methoxy-4′-nitro- and 4′-amino-3′-methoxyflavone inhibit TCDD-induced CYP1A1 gene expression in MCF-7 human breast cancer cells and the inhibitory effects by 3′,4′-substituted flavones are remarkably dependent on the structure and position of the substituents. 3′-Methoxy-4′-nitroflavone (**1**) is a pure Ah receptor antagonist and results obtained with flavones **1** and **2** indicate that both compounds partially inhibit formation of the nuclear Ah receptor complex. The failure of flavones **1** and **2** to block formation of an early eluting specifically bound nuclear Ah receptor complex suggests that MCF-7 cells express a form of the Ah receptor complex which exhibits differential (low) competitive binding with flavones **1** and **2** and is inactive as a transcriptional enhancer for induction of CYP1A1 in this cell line. These compounds will be utilized in the future as probes to further characterize multiple forms of the Ah receptor complex.

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Modulation of Luminol-Dependent Chemiluminescence of Murine Macrophages by Flavone and its Synthetic Derivatives

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Modulation of Luminol-dependent Chemiluminescence of Murine Macrophages by Flavone and Its Synthetic Derivatives

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Summary

The effect of flavone (CAS 525-82-6, 2-phenylbenzopyran-4-one, 1), flavone-8-acetic acid (CAS 87626-55-9, FAA, 2) and 10 substituted flavones on the luminol-dependent chemiluminescence of murine macrophages was studied in vitro. The synthetic derivatives were variously substituted with halo, nitro, amino, hydroxy and methoxy substituents in the 3' and 4' positions. Chemiluminescence was used in this study as an indicator for the production of reactive oxygen species by macrophages, stimulated in vitro by phorbol myristate acetate (PMA). All flavones except FAA (2) showed more than 20% inhibition at 10 μmol/l or 100 μmol/l. 3'-Amino-4'-hydroxyflavone (8) was the most potent inhibitor. The IC_{50}s for inhibition of chemiluminescence were 4.2 ± 1.1 μmol/l, 5.0 ± 1.0 μmol/l and 3.3 ± 1.4 μmol/l for resident, elicited and LPS-Poly I:C-primed macrophages, respectively. Small but statistically significant enhancements of chemiluminescence were caused by low concentrations of flavone (1), FAA (2) and 4'-methoxyflavone (6). These results suggest that modulation of the chemiluminescent capacity of macrophages depends on the nature of the substituents and the concentration of the flavones.

Zusammenfassung

Regulierung der Lumino-abhängigen Chemilumineszenz in Maus-Makrophagen durch Flavon und dessen synthetische Derivate

Flavon (CAS 525-82-6, 2-Phenylbenzopyran-4-on, 1), Flavon-8-essigsäure (CAS 87626-55-9, FAA, 2) und 10 substituierte Flavone wurden auf ihren Einfluß auf die Luminol-abhängige Chemilumineszenz hin untersucht. Die substituierten Flavone, welche an den Positionen 3 und 4 Halo-, Nitro-, Amino-, Hydroxy- oder Methoxy-Gruppen besitzen, wurden synthetisch gewonnen. Chemilumineszenz, die in vitro mit Hilfe von Phorbolmyristylacetat (PMA) stimuliert wurde, diente als Indikator für die Entstehung von reaktiven Sauerstoffprodukten in den Makrophagen. Alle Flavone, mit Ausnahme von FAA (2), zeigten bei einer Konzentration von 10 oder 100 μmol/l eine 20%ige Inhibierung. 3'-Amino-4'-hydroxyflavon (8) war von allen der wirkungsvollste Inhibitor. 50%ige Hemmung (IC_{50}) wurde bei folgenden Konzentrationen hervorgerufen: 4.2 ± 1.1 μmol/l für nicht stimulierte Makrophagen, 5.0 ± 1.0 μmol/l für stimulierte Makrophagen, 3.3 ± 1.0 μmol/l für mit LPS-Poly I:C vorbehandelte Makrophagen. Eine geringe Verstärkerung der Chemilumineszenz konnte bei Flavon (1), FAA (2) und 4'-Methoxyflavon (6) beobachtet werden. Diese Ergebnisse zeigen, daß die Regulierung der Chemilumineszenzstärke in Makrophagen möglich ist, und ferner abhängig ist von der Art und der Konzentration der Flavone.

Key words: *3'-Amino-4'-hydroxyflavone · CAS 87626-55-9 · Flavone-8-acetic acid · Flavones · Macrophages, modulation of chemiluminescence*

1. Introduction

Various flavones (2-phenylbenzopyran-4-one derivatives) have a wide range of effects on biological systems [1–3] and some naturally occurring flavones show anticancer activity against certain experimental tumours [4–7]. Recent reports have indicated the potential use of synthetic flavonoids, such as flavone-8-acetic acid (CAS 87626-55-9, FAA, LM-975, NSC-347512, 2) and its analogues, in the treatment of some solid tumours in experimental models [8–10]. FAA is more effective against slow-growing solid tumours (e.g. murine colon adenocarcinoma 38) than against rapidly proliferating leukaemias and lymphomas (e.g. L1210 and P388) [11, 12] and has been reported to have effects on shutting down blood flow and inducing haemorrhagic necrosis [15] in experimental murine solid tumours. The ability of FAA and analogues to stimulate natural killer cells [16] and T cells [17] has also been reported. FAA increases the direct cytotoxicity of murine macrophages in vitro against tumour targets [18], stimulates the formation of nitric oxide [19] and modulates production of superoxide anion (O_2^-) in activated macrophages [20]. Several synthetic aminoflavones also show inhibitory activity against protein kinases [21, 22]. These activities in normal and tumour-bearing mice and in human patients suggest that there may be potential for further chemical studies to develop flavones as chemotherapeutic drugs with improved antitumour activity and which may also function as biological response modifiers.

Upon stimulation with both soluble and particulate matter, oxidative metabolism is stimulated in the macrophage, resulting in the respiratory burst which is accompanied by activation of an NADPH oxidising enzyme. This enzyme catalyses the reduction of molecular oxygen to O_2^- and the burst is paralleled by consumption of oxygen. Rapid dismutation of O_2^- gives hydrogen peroxide (H_2O_2). These toxic metabolites are important for the tumouricidal and microbicidal activities of macrophages [23–25]. The presence of reactive oxygen metabolites can be inferred from the light emitted (chemiluminescence) during their reaction with the easily oxidisable substance luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) [26].

The purpose of the present study was to evaluate quantitatively the activity of flavones on the chemiluminescent activity of murine macrophages. A series of flavones variously substituted in the 3' and 4' positions was prepared for evaluation of their activity towards macro-

phages. The assay technique evaluates the luminol-dependent chemiluminescence of macrophages, using phorbol myristate acetate (PMA) as a stimulant for oxygen metabolism through the activation of protein kinase C (PKC) [27].

Abbreviations

FAA, flavone-8-acetic acid; $O_2^{\cdot-}$, superoxide anion; PMA, phorbol myristate acetate; PKC, protein kinase C; HBSS, Hanks' balanced salt solution; PBS, phosphate buffered saline solution DMSO, dimethylsulphoxide; LPS, lipopolysaccaride; Poly I:C, polyinosinic-polycytidylic acid potassium salt; IFNs, interferons; IL, interleukin; TNF, tumour necrosis factor.

2. Methods

2.1. Chemicals

FAA (2) was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, USA, through the courtesy of Dr. K. Paull. Other flavones (1, 3–12) were synthesised as previously described [21]. HBSS without phenol red and PBS were purchased from GIBCO Life Technologies Ltd. (Paisley, UK). PMA and DMSO were purchased from Sigma Chemical Company (St. Louis, MO, USA). Luminol was bought from LKB (Turku, Finland). Thioglycollate broth and phenol red solution (0.5 % aqueous solution of the sodium salt) were purchased from Serva Chemicals (Heidelberg, Germany). E. coli serotype 0127:B8 LPS was purchased from Calbiochem (La Jolla, CA, USA). Poly I:C was purchased from Biochemicals Inc. (Milwaukee, WI, USA). Monoclonal antibody anti-mouse Thy-1,2 fluorescein conjugate was purchased from Becton Dickinson Systems (Mountain View, CA, USA).

2.2. Isolation and culture of mouse peritoneal exudate macrophages

Peritoneal exudate cells were isolated from BALB/c mice (8–12 weeks of age). Mice were sacrificed by cervical dislocation and cells were collected by washing the peritoneum with PBS (5 ml). Resident macrophages were collected from untreated animals. Thioglycollate-elicited macrophages were collected from mice which had been injected 4 days previously with thioglycollate broth (1 ml per mouse). LPS-Poly I:C-stimulated macrophages were obtained from mice which had been injected 18 h previously with 1 ml of a solution of LPS plus Poly I:C (100 µg Poly I:C plus 0.1 µg LPS) in PBS. Adherent cells were collected by plating the cells in culture medium (1 ml) in test tubes or 24-well plates and incubating for 2 h at 37 °C under 95 % air/5 % CO_2 and 100 % humidity. Non-adherent cells were removed with the supernatant and by three further vigorous washings with PBS. The adherent population was judged to be 92 ± 2 % macrophages by differential counts of May-Grunwald/Giemsa stained cells and less than 1 % T-lymphocytes by flurescence microscopy after fluorescent antibody labelling with anti Thy 1,2 antibody.

Monolayers of macrophages were tested for viability in 24-well plates (Falcon, Becton Dickinson, Lincoln Park, NJ, USA) containing one glass cover slip per well. Viability was greater than 96 ± 2 % as determined by exclusion of neutral red. The same staining was also used for evaluating the cytotoxic efficiency of the tested flavones. All flavones were dissolved in DMSO. DMSO at the maximum concentration (1 %) did not modify the viability of cells or the chemiluminescence in the macrophage cultures in vitro.

2.3. Chemiluminescence

Chemiluminescence was measured as previously described [28], using a special low noise-count-rate photomultiplier (9514S, EMI, Middlesex, UK). The bi-alkali photocathode of this photomuliplier has a spectral response of 300 to 660 nm. The luminescent light was measure in 3.5 ml test tubes. The sample volume was usually 1 ml. The reactions were initiated by dispensing aliquots of solutions of luminol (110 µmol/l) to macrophages (2×10^6) and flavones in HBSS in test tubes. The resting intensity of chemiluminescence was recorded after 5 min. PMA solution was then added to give a final concentration of 0.8 µmol/l. The light emitted was then recorded continuously for 15 min. The intensity of the chemiluminescence was determined by measuring counts/min and by calculating the area under the chemiluminescence intensity curve (integral chemiluminescence). The percent inhibition of chemiluminescence, as compared to controls, was calculated according to the formula:

$$\text{Inhibition \%} = 100 \times (N_c - N_t)/N_c$$

where N_c values were the counts per 15 min for the control and N_t values were the counts per 15 min in the presence of the flavones. A negative value indicates stimulation. The final concentrations of the flavones were 1, 10, 100 and 1000 µmol/l (the latter concentration was only used for FAA).

3. Results

Three states of differentiation of murine peritoneal macrophages (resident, elicited and activated) are generally recognised. Resident cells were collected from untreated animals. Elicited macrophages were prepared from mice which had been injected 4 days previously with thioglycollate broth. LPS-Poly I:C-primed macrophages (activated) were obtained from mice which had been injected 18 h previously with a solution of LPS and Poly I:C. The kinetic profile of chemiluminescence of macrophages pre-exposed to luminol and (5 min later) to PMA (0.8 µmol/l) is presented in Fig. 1. This variation in kinetics of chemiluminescence was observed when different macrophages were used. Qualitatively similar profiles were observed with resident and elicited macrophages. The response curves demonstrated that the resident and elicited macrophages caused a slow rising chemiluminescence curve without a defined peak after dispersion of luminol. LPS and Poly I:C stimulated maximal production of chemiluminescence by the macrophages. The addition of luminol resulted in a slight but significant increase in the count rate, due to activation of the cells by LPS and Poly I:C before harvesting them in vivo. The increase in count rate after the addition of PMA was significantly larger and peaked within 4 to 5 min. The production of chemiluminescence by peritoneal macrophages is presented in Table 1.

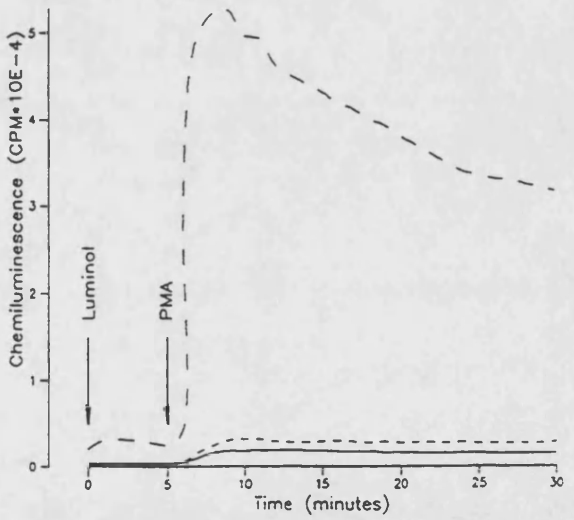


Fig. 1: Time course of chemiluminescence emitted from macrophages exposed to 0.8 µmol/l of PMA. ——— Resident, - - - - elicited, — — — LPS-Poly I:C-primed.

Table 1: Production of chemiluminescence by peritoneal macrophages.

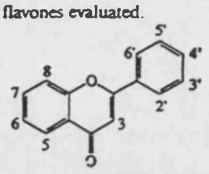
Macrophages	Chemiluminescence response (integral counts)[a]	
	Time (min) after adding luminol[b]	
	0–5	6–20
Resident	167 ± 8	2386 ± 227
Elicited	193 ± 14	4122 ± 727
Activated	1446 ± 278	51667 ± 9387

[a] Data are expressed as the mean values of 4–6 experiments ± SD.
[b] PMA was added 5 min after the addition of luminol.

The structures of the flavones used in this work are shown in Table 2. The level of inhibition of all twelve compounds tested is presented in Tables 3–5. Of these, six compounds inhibited chemiluminescence at all concentrations tested (3, 8–12). 3'-Amino-4'-hydroxyflavone (8) was the most potent inhibitor. The concentrations leading to 50 % inhibition (IC_{50}) of chemiluminescence by this compound were 4.2 ± 1.1 μmol/l, 5.0 ± 1.0 μmol/l and 3.3 ± 1.4 μmol/l for resident, elicited and LPS plus Poly I:C – primed macrophages, respectively. Since no significant difference in the viability of cells was observed between cultures exposed to PMA alone (92 ± 2 % viable) and to PMA and the highest concentrations tested for each of the agents, inhibition of production of chemiluminescence is unlikely to be due to toxicity of the compounds.

PMA-stimulated production of chemiluminescence was enhanced to a small but statistically significant extent in the presence of low (1 μmol/l) concentrations of flavone (1), FAA (2) and 4'-methoxyflavone (6), independently of the macrophages used. However, PMA-stimulated production of chemiluminescence was progressively inhibited in the presence of increasing concentrations of these agents.

Table 2: Structures of flavones evaluated.



Flavone	Name	3'-Substituent	4'-Substituent	8-Substituent
1	flavone	H	H	H
2	flavone-8-acetic acid	H	H	CH_2COOH
3	4'-aminoflavone	H	NH_2	H
4	4'-bromoflavone	H	Br	H
5	4'-chloroflavone	H	Cl	H
6	4'-methoxyflavone	H	OCH_3	H
7	3'-amino-4'-methoxyflavone	NH^2	OCH_3	H
8	3'-amino-4'-hydroxyflavone	NH_2	OH	H
9	4'-chloro-3'-nitroflavone	NO_2	Cl	H
10	3',4'-dichloroflavone	Cl	Cl	H
11	3'-methoxy-4'-nitroflavone	OCH_3	NO_2	H
12	4'-methoxy-3'-nitroflavone	NO_2	OCH_3	H

Table 3: Effects of flavones on chemiluminescence in murine resident macrophages by PMA.

Flavone	Inhibition (%)[a]			
	Concentration (μmol/l)			
	1	10	100	1000
1	−14 ± 2	20 ± 2	69 ± 3	n.d.
2	2 ± 1	−1 ± 1	8 ± 2	58 ± 2
3	0.3 ± 0.2	20 ± 1	66 ± 3	n.d.
4	14 ± 2	33 ± 2	n.d.	n.d.
5	11 ± 3	32 ± 2	n.d.	n.d.
6	−13 ± 2	23 ± 2	n.d.	n.d.
7	−6 ± 2	22 ± 3	n.d.	n.d.
8	34 ± 2	61 ± 3	81 ± 4	n.d.
9	36 ± 2	48 ± 1	64 ± 3	n.d.
10	4 ± 1	16 ± 2	23 ± 2	n.d.
11	8 ± 1	18 ± 1	21 ± 3	n.d.
12	4 ± 2	13 ± 1	n.d.	n.d.

[a] Results are expressed as mean ± SD, n = 3–5. n.d.: not determined.

Table 4: Effects of flavones on chemiluminescence induced in murine elicited macrophages by PMA.

Flavone	Inhibition (%)[a]			
	Concentration (μmol/l)			
	1	10	100	1000
1	−11 ± 2	7 ± 3	74 ± 3	n.d.
2	5 ± 1	−6 ± 2	9 ± 2	59 ± 3
3	0.2 ± 0.1	8 ± 2	75 ± 2	n.d.
4	16 ± 2	36 ± 2	n.d.	n.d.
5	31 ± 3	32 ± 2	n.d.	n.d.
6	−2 ± 1	22 ± 3	n.d.	n.d.
7	11 ± 2	22 ± 1	69 ± 3	n.d.
8	30 ± 2	55 ± 3	85 ± 2	n.d.
9	35 ± 2	47 ± 2	56 ± 4	n.d.
10	10 ± 2	20 ± 3	30 ± 3	n.d.
11	7 ± 2	12 ± 3	22 ± 4	n.d.
12	9 ± 1	17 ± 2	n.d.	n.d.

[a] Results are expressed as mean ± SD, n = 3–5. n.d.: not determined.

Table 5: Effects of flavones on chemiluminescence induced in murine LPS-Poly I:C macrophages by PMA.

Flavone	Inhibition (%)[a]			
	Concentration (μmol/l)			
	1	10	100	1000
1	−6 ± 2	−8 ± 3	55 ± 3	n.d.
2	−3 ± 1	−8 ± 3	−6 ± 4	35 ± 6
3	6 ± 2	8 ± 3	70 ± 3	n.d.
4	−10 ± 4	25 ± 2	n.d.	n.d.
5	−11 ± 2	13 ± 2	n.d.	n.d.
6	−7 ± 3	16 ± 3	n.d.	n.d.
7	−2 ± 1	2 ± 1	65 ± 3	n.d.
8	36 ± 2	52 ± 2	93 ± 5	n.d.
9	33 ± 1	30 ± 4	35 ± 5	n.d.
10	5 ± 1	4 ± 3	6 ± 1	n.d.
11	2 ± 1	12 ± 2	14 ± 3	n.d.
12	0.2 ± 0.1	20 ± 4	n.d.	n.d.

[a] Results are expressed as mean ± SD, n = 3–5. n.d.: not determined.

4. Discussion

The generation of chemiluminescence by mouse peritoneal macrophages is an indication of the formation of reactive oxygen species. The chemiluminescence is dependent on the production of species such as $O_2^{\cdot-}$, H_2O_2, $^1O_2$ and $HO^{\cdot}$. The production of such highly reactive entities in macrophages is a result of the activation of the respiratory burst. In this report, we show that LPS plus Poly I:C stimulated the activity of macrophages in vivo. Production of chemiluminescence stimulated by LPS plus I:C was significantly enhanced in the presence of luminol. Induction of macrophage oxidative activity in vivo is a multi-step process requiring both a priming (e.g. IFNs) and a triggering (e.g. LPS) signal. Double-stranded polyribonucleotides such as Poly I:C are potent inducers of IFNs both in vivo and in vitro [29, 30]. Additionally, Poly I:C induces the secretion of TNF-α by macrophages [31]. LPS is one of the most powerful activators of macrophages, which then produce a variety of active substances such as TNFs, IL-1 and IL-6 [31, 32]. Macrophages of various species, including man, give a respiratory burst upon exposure to phorbol esters; indeed, PMA is one of the most potent activators of NADPH oxidase known [27]. Primed peritoneal macrophages show higher capacities than resident cells to generate $O_2^{\cdot-}$ upon stimulation by PMA. Unlike resident murine macrophages, macrophages derived from murine bone marrow generate $O_2^{\cdot-}$ upon exposure to zymosan but not to PMA. The ability of macrophages derived from bone marrow to respond to PMA is restored, at least in part, by treatment with LPS or the cytokines TNF-α, IFN-γ and IL-1α [33]. Thus, the responsiveness of resident peritoneal macrophages to PMA may be the result of exposure to cytokines in vivo.

We have shown that the resident and elicited peritoneal macrophages generated chemiluminescence upon expo-

sure to PMA and this may reflect the response of a subpopulation of resident macrophages which arises as a result of chronic low level stimulation by endogenously produced endotoxin in BALB/c mice. The present experiments demonstrate that the PMA-stimulated generation of chemiluminescence in vitro from peritoneal macrophages stimulated with LPS and Poly I:C (in vivo) was enhanced to a small but significant extent in the presence of low concentrations of flavones **1**, **4–7** at 1 µmol/l and by FAA (**2**) at 1, 10, 100 µmol/l. At higher concentrations of these agents, PMA-stimulated generation of chemiluminescence was inhibited in a concentration-dependent manner. Flavones **3**, **8–12** inhibited the chemiluminescence at all concentrations tested, again in a dose-dependent way. A similar activity has been shown for FAA and xanthenone acetic acid analogues with PMA-stimulated production of superoxide measured as reduction of ferricytochrome c that is inhibitable by superoxide dismutase [20]. The cellular mechanisms of the effect are not known but could be related to the various biological effects of the flavones: 1. antioxidant properties [28, 34]; 2. inhibition of many enzymes, including NADPH oxidase [35], cyclo-oxygenase and lipoxygenase [36, 37]; 3. inhibition of cellular enzymes involved in signal transduction [21, 22, 38, 39].

The flavones could inhibit chemiluminescence of macrophages by inhibiting the generation or release of radicals from the cells. In a previous study, we demonstrated that some flavones modulated luminol-dependent chemiluminescence of neutrophils [40]. In contrast, 3-hydroxyflavones are well known to be potent inhibitors of the chemiluminescence of neutrophils [41]. Various flavones, e.g. kaempferol (3,4',5,7-tetrahydroxyflavone), morin (2',3,4'-5,7-pentahydroxyflavone) and quercetin (3,3',4',5,7-pentahydroxyflavone) inhibit the respiratory burst in neutrophils induced by soluble and particulate stimuli [35].

There appears to be no clear structure-activity relationship amongst the eleven synthetic flavones for inhibition of the respiratory burst in resident, elicited and activated macrophages, although there is a correlation between activity of any one compound on all three macrophage types. There is some indication that polarity of the substituents has importance, with flavones **3**, **7**, **8** which, bear polar, hydrogen-bonding substituents being good inhibitors and flavones **10–12** with non-polar substituents being weak inhibitors. The exception to this generalisation is 4'-chloro-3'-nitroflavone (**9**) which is a relatively strong inhibitor. Similarly, there is a tendency for flavones bearing electron-donating substituents (e.g. **3**, **7**, **8**) to have potent activity and those having electron-withdrawing groups (e.g. **11**, **12**) to show poor activity but again there is an exception in that the relatively electron-deficient 4'-chloro-3'-nitroflavone (**9**) is relatively potent.

In the present study, we have shown 3'-amino-4'-hydroxyflavone (**8**) to be the most potent inhibitor. This compound also reduced the amount of chemiluminescence generated by the horseradish peroxidase mediated oxidation of luminol by hydrogen peroxide (IC_{50} = 5.5 ± 0.5 µmol/l) (Król, W., Czuba, Z. P., Threadgill, M. D., unpublished results). Thus some of the activity of this flavone may be due to antioxidant reactivity, although this could not be so for the relatively electron-deficient analogue **9**. The mechanism by which these agents inhibit NADPH oxidase may involve interference with PKC-mediated protein phosphorylation [42]. It is also noteworthy that the concentration of the active compound is a major determinant of its activity; it has already been shown that while low concentrations of quercetin stimulate activation of PKC, high concentrations inhibit it [38, 39]. This feature may be an important determinant in the immunotropic activity of flavones where signal transmission processes across cell membranes play an important rôle in their biological significance. Further studies on the biological effects of the active flavones are in progress.

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Monofunctional Electrophilic and Nucleophilic Derivatives of *Meso*-Tetraphenylporphyrin for Attachment to Peptides

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Monofunctional Electrophilic and Nucleophilic Derivatives of *meso*-Tetraphenylporphyrin for Attachment to Peptides

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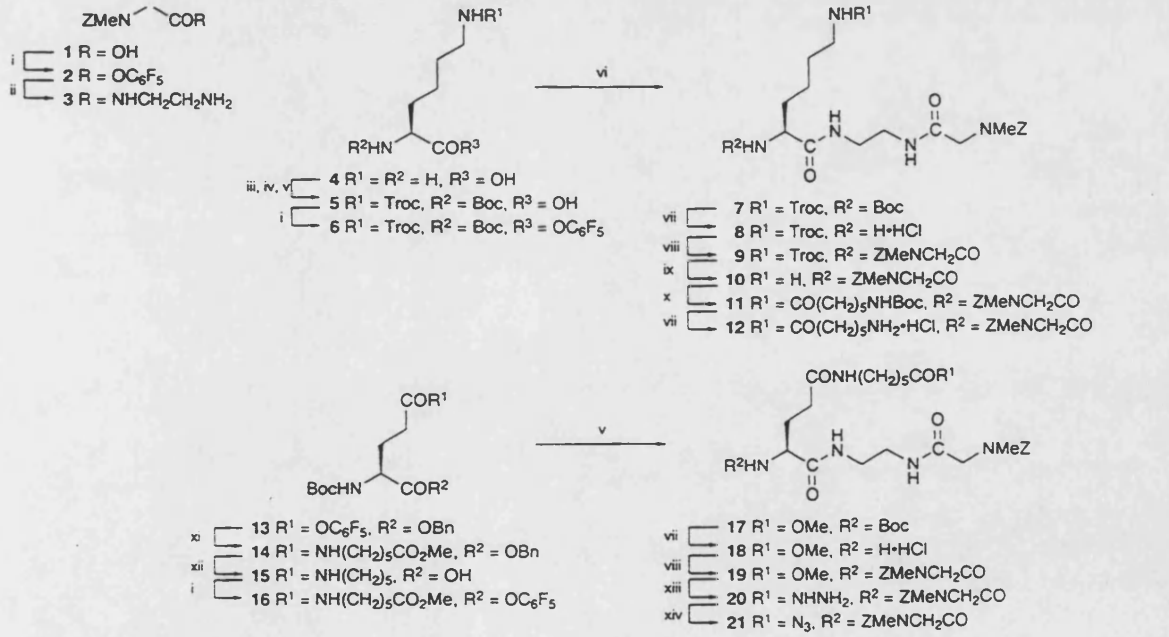
4-Nitrophenyl *N*-[4-(10,15,20-triphenylporphyrin-5-yl)phenyl]carbamate and 5-[4-(*N*-glycylamino)phenyl]-10,15,20-triphenylporphyrin have been synthesised from a readily prepared monofunctionalised porphyrin; they couple efficiently with the side-chains of extended lysyl and glutamyl peptide derivatives, respectively.

Porphyrins alone or linked to polymers and other targetting moieties have important roles in diagnosis and therapy of cancer. For example, the so-called 'haematoporphyrin derivative' and other porphyrins act as photosensitisers for conversion of triplet oxygen to singlet oxygen in photodynamic therapy[1] and porphyrins are known to accumulate selectively in some types of tumour tissue,[2] leading to prospects of their use as targetting groups. Porphyrinatomanganese complexes are used as contrast-enhancing agents in magnetic resonance imaging (MRI), owing to their high molar relaxivities in aqueous solution.[3] Attachment of metalloporphyrin and other agents to a soluble polymer enhances molar relaxivity and thus effectiveness as a MRI contrast agent.[4]

Significant difficulty in preparing mono-functionalised porphyrins in a controlled manner is caused by the fact that the most readily available naturally-occurring porphyrins, such as protoporphyrin IX and mesoporphyrin II, carry more than one identical electrophilic or nucleophilic functional group. The classical Adler–Rothemund[5] procedure for synthesis of *meso*-tetraarylporphyrins from arylaldehydes and pyrrole proceeds in <20% yield and mono-Ar-functionalised porphyrins are only obtained in very low yields by separation of statistical mixtures of porphyrins formed from mixtures of aldehydes,[6] whereas the recently-reported Heck coupling[7] needs several synthetic steps to prepare the starting 5,15-diphenyl-10-iodoporphyrin. To obviate these problems of synthesis of mono-Ar-substituted tetraphenylporphyrins, Kruper *et al.*[8] developed an efficient mononitration and subsequent reduction of the readily-available *meso*-tetraphenylporphyrin. We now report our exploitation of this weakly nucleophilic amine in generating reactive electrophilic and nucleophilic monofunctional porphyrins for attachment to side-chain extended α,ω-bis(methylamino) peptides. Polymers derived from the latter will be of use in MRI.

α,ω-Bis[benzyloxycarbonyl(methyl)amino] peptides with carboxylic acid derivatives and primary amines in side-chains of the same length were built up as shown in Scheme 1. *N*-(Benzyloxcarbonyl)sarcosine **1**[9] was converted to its pentafluorophenyl (PFP) active ester **2**† and this was added to a 20-fold excess of ethane-1,2-diamine to set up the protected sarcosine aminoethylamide **3**† as the sequence inverting unit for the C-termini of the peptides.

Orthogonal protection of the α- and ε-amines of L-lysine **4** was required for elaboration of the peptide chain and of the side-chain. This was achieved by complexation with copper(II), selective acylation of the ε-amine with 2,2,2-trichloroethyl chloroformate, decomplexation and acylation of the α-amine with di-*tert*-butyl dicarbonate in a two-phase system, in a modification of the method of Yajima *et al.*[10] The resulting BocLys(Troc)OH **5**† was converted to the PFP active ester **6**,† prior to coupling with **3** to afford the fully orthogonally



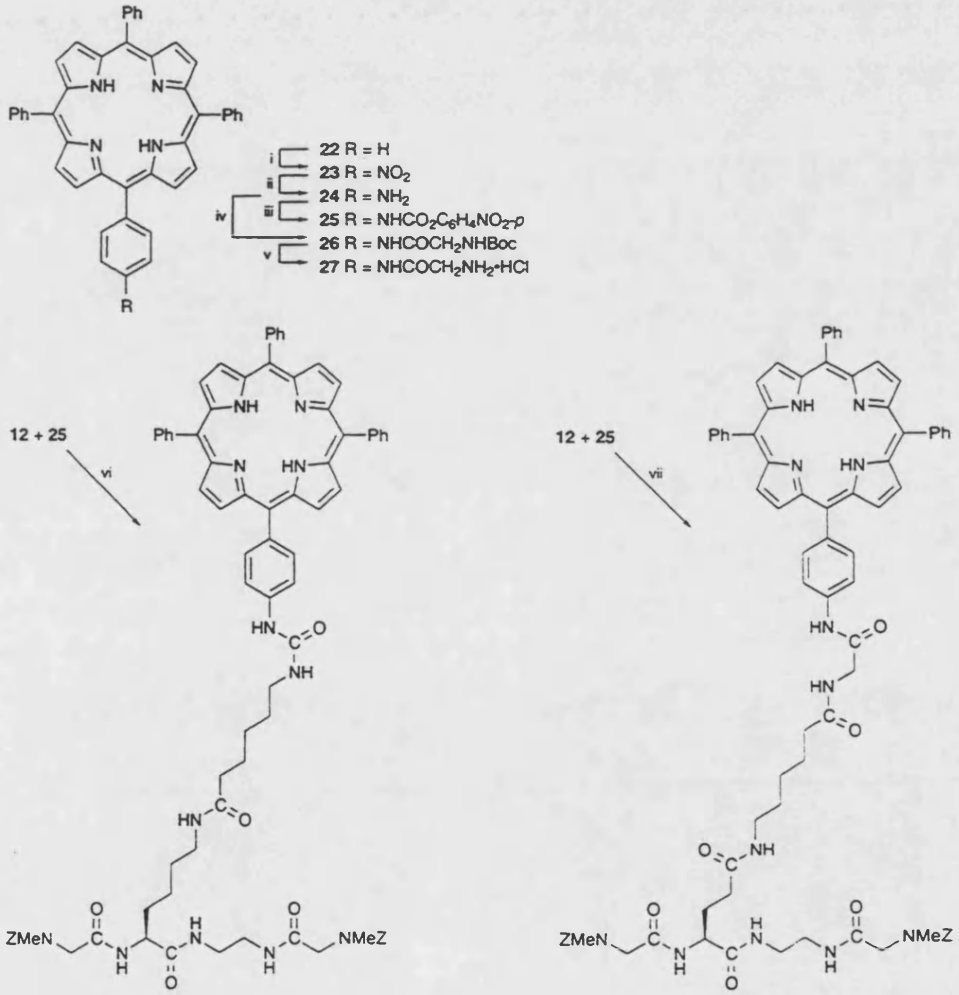
Scheme 1 Synthesis of extended sequence-inverted peptides **12** and **21**. Troc = 2,2,2-trichloroethoxycarbonyl. *Reagents and conditions*:†† i, C_6F_5OH, DCC, EtOAc, 0 °C, 20 h, 90–95%; ii, $H_2NCH_2CH_2NH_2$ (20 × excess), CH_2Cl_2, 3 h, 82%; iii, $CuCO_3$, H_2O, reflux, 3 h, then TrocCl, Na_2CO_3, H_2O, 0 °C, 20 h; iv, $Na^+{}_2EDTA^{2-}$, H_2O, reflux, 2 h; v, Boc_2O, Et_3N, H_2O, dioxan, 3 d, 58% from **4**; vi, **3**, Pr^i_2NEt, CH_2Cl_2, 85%; vii, HCl, CH_2Cl_2, 1 h, quant.; viii, **2**, Pr^i_2NEt, DMAP, CH_2Cl_2, 4 d, 87%; ix, Zn, MeOH, reflux, 5 h, 83%; x, $BocNH(CH_2)_5CO_2C_6F_5$, Pr^i_2NEt, DMAP, CH_2Cl_2, 6 d, 55%; xi, $H_2N(CH_2)_5CO_2Me{\cdot}HCl$, Pr^i_2NEt, DMAP, CH_2Cl_2, 7 d, 89%; xii, H_2, Pd/C, tetrahydrofuran, 3 h, quant; xiii, $N_2H_4{\cdot}H_2O$, MeOH, 40 °C, 8 h, quant; xiv, Bu^tONO, DMF, THF, dioxan, HCl, −20 °C, 50 min, then Pr^i_2NEt, −60 °C (this solution was taken forward for reaction with **27**, Scheme 2).

protected inverted-sequence peptide **7**.† The peptide chain was completed by acidic removal of the Boc group, giving the salt **8**,† and acylation with one further equivalent of **2**. With the inverted sequence of the peptide now complete, attention was turned to extension of the length of the side-chain of **9**.† The ε-amine **10**† was revealed by selective reductive removal of the Troc group (zinc dust in methanol). Acylation with pentafluorophenyl 6-(*tert*-butoxycarbonylamino)hexanoate[11] gave the orthogonally protected peptide derivative **11**.† Deprotection (HCl) afforded the target inverted-sequence peptide **12**† with the extended primary amine side-chain for coupling with an appropriate monofunctional porphyrin electrophile.

In the assembly of a corresponding inverted-sequence peptide with an extended activated carboxylic acid side-chain, the extension was performed prior to construction of the peptide to avoid problems of formation of pyroglutamates. Acylation of the spacer unit, methyl 6-aminohexanoate, with Boc glutamic acid α-benzyl ester γ-PFP ester **13**[12] gave the extended derivative **14**.† The α-carboxylic acid **15**† was revealed by selective hydrogenolysis of the benzyl ester. In a series of steps similar to those used for building the inverted-sequence lysine peptide, this carboxylic acid was activated as the PFP ester **16**† and coupled with the sequence-inverting unit **3** to afford **17**.† Again, selective acidolytic deprotection revealed the Glu α-amine **18**. Coupling with **3** afforded the target inverted-sequence peptide **19**† with the extended carboxylic acid side-chain protected as the methyl ester. This ester resisted selective hydrolysis under both basic and acidic conditions but succumbed to hydrazinolysis, giving the hydrazide **20**.† From this, the acyl azide **21**††‡ was formed by reaction with *tert*-butyl nitrite under acidic conditions, other nitrosating agents (*e.g.* sodium nitrite) being either inefficient or destructive. This provides an active acylating function for reaction with an appropriate porphyrin nucleophile.

The monoaminophenylporphyrin **24** was prepared in 46% overall yield in two steps from *meso*-tetraphenylporphyrin **22**, in a modification of the method of Kruper *et al.* (Scheme 2).[8] The corresponding isocyanate would represent a potent electrophile for reaction with the extended lysine derivative **12** but **24** reacted slowly with phosgene, giving mainly the corresponding *N,N'*-bis(tetraphenylporphyrinyl)urea. However, the amine **24** was acylated smoothly§ by 4-nitrophenyl chloroformate, giving the carbamate **25**, a synthon for the required isocyanate. Treatment of **25** under mildly basic conditions generated the isocyanate which coupled *in situ*§ with the extended lysine derivative **12**, giving the protected porphyrinyl peptide derivative **28**¶ in good yield.

The arylamine **24** was found to be a remarkably weak nucleophile, reacting with succinic anhydride only after a prolonged period at elevated temperature and not reacting with



Scheme 2 Activation of tetraphenylporphyrinamine **24** as an electrophile and as a nucleophile; coupling with extended sequence-inverted peptides **12** and **21**. *Reagents and conditions*:†† i, fuming HNO_3, $CHCl_3$, 5 h, 55%; ii, $SnCl_2$, conc. aq. HCl, 80 °C, 2 h, 84%; iii, 4-nitrophenyl chloroformate, Pr^i_2NEt, $CHCl_3$, 20 h, then chromatography, 67%; iv, $BocGlyOC_6F_5$, Pr^i_2NEt, DMAP, $CHCl_3$, 46 h, 95%; v, HCl, CH_2Cl_2, 1 h, quant; vi, Pr^i_2NEt, DMAP, CH_2Cl_2, 20 h, 82%; vii; Pr^i_2NEt, DMAP, $CHCl_3$, 2 h, 58%.

peptide active esters or with the acyl azide **21**. Much greater nucleophilicity is required for efficient coupling with peptide derivatives under mild conditions. To introduce a primary aliphatic amine as a more potent nucleophile, the arylamine was acylated by treatment‖ with a two-fold excess of the PFP active ester of N-Boc-glycine at 40 °C, forming **26**. The primary aliphatic amine **27** was revealed by deprotection‖ with hydrogen chloride. This more reactive nucleophile then coupled efficiently‖ with the extended peptide derivative acyl azide **21**, giving the porphyrinyl peptide derivative **29**.**

The monoaminotetraphenylporphyrin **24** is thus demonstrated to be a readily accessible monofunctionalised porphyrin which can be converted straightforwardly into a reactive electrophile and a reactive unhindered nucleophile which should have general utility in controlled attachment of porphyrins to peptides, polymers and other molecules.

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Footnotes

† All novel compounds were characterised by ^{1}H NMR and by FAB MS and were shown to be pure by TLC. Target compounds and major intermediates were also characterised by high resolution FAB MS.

‡ Acyl azide **21** was prepared and used without purification, to avoid Curtius rearrangement.

§ *Method*: Compound **24** (5.70 g, 9.25 mmol) was stirred with 4-nitrophenyl chloroformate (1.86 g, 9.25 mmol) and Pr^i_2NEt (1.19 g, 9.25 mmol) in $CHCl_3$ (50 cm^3) for 20 h. Chromatography gave **25** (4.85 g, 67%) as a purple glass. Compound **12** (385 mg, 530 μmol) and **25** (520 mg, 670 μmol) were stirred with Pr^i_2NEt (205 mg, 1.6 mmol) and DMAP (10 mg) in CH_2Cl_2 (10 cm^3) for 20 h. Chromatography gave **28** (593 mg, 82%) as a purple glass.

¶ *Spectroscopic data* for **28**: δ ($CDCl_3$) −2.78 (2 H, s, porphyrin 21,23-H_2), 1.2–1.7 (10 H, m, Lys β, γ-H_4 + $NCH_2CH_2CH_2CH_2CO$), 2.12 (2 H, br, CH_2CH_2CO), 2.93 (3 H, s, NCH_3), 2.99 (3 H, s, NCH_3), 3.0–3.3 (8 H, Lys ε-H_2 + $NCH_2CH_2CH_2$ + NCH_2CH_2N), 3.77 (2 H, m, Sar-H_2), 3.86 (2 H, m, Sar'-H_2), 4.36 (1 H, m, Lys α-H), 5.06 (3 H, s) and 5.07 (1 H, s) (2 × $PhCH_2O$), 5.75 (1 H, br, NH), 5.83 (1 H, br, NH), 6.39 (1 H, br, NH), 6.57 (1 H, br, NH), 6.94 (1 H, br, NH), 7.24 (10 H, br s, 2 × benzyloxy Ph-H_5), 7.37 (1 H, br, NH), 7.69 (11 H, m, 3 × porphyrin-Ph 3,4,5-H_3 + porphyrin-C_6H_4N 2,6-H_2), 8.06 (2 H, d, *J* 8.4 Hz, porphyrin-C_6H_4N 3,5-H_2), 8.15 (6 H, m, 3 × porphyrin-Ph 2,6-H_2), 8.79 (2 H, d *J* 4.7 Hz, porphyrin 3,7-H_2), 8.82 (4 H, s, porphyrin 12,13,17,18-H_4), 8.87 (2 H, d, *J* 5.1 Hz, porphyrin 2,8-H_2); *m/z* (FAB) 1367.6384 (M + H) ($C_{81}H_{83}N_{12}O_9$ requires 1367.6406).

‖ *Method*: Compound **24** (5.00 g, 8.16 mmol) was stirred with Boc-$GlyOC_6F_5$ (5.58 g, 16.3 mmol), Pr^i_2NEt (2.32 g, 18.0 mmol) and DMAP (50 mg) in $CHCl_3$ (100 cm^3) for 46 h at 40 °C. Chromatography gave **26** (6.00 g, 95%) as a purple glass. This compound (2.24 g, 2.9 mmol) was treated with excess HCl in CH_2Cl_2 (100 cm^3) for 1 h. The solvent and excess reagent were evaporated to give **27** (2.10 g, quantitative). *tert*-Butyl nitrite (0.22 cm^3) in THF (1.75 cm^3) was added to **21** (1.50 mg, 2.07 mmol) in DMF (3.0 cm^3) and HCl in 1,4-dioxan (4.0 mol dm^{-3}, 1.86 cm^3) at −20 °C. The mixture was stirred for 2 h. Pr^i_2NEt (1.06 g) was added at −60 °C, followed by **27** (2.09 g, 2.9 mmol) and Pr^i_2NEt (1.12 g, 18.7 mmol) in $CHCl_3$ (30 cm^3). The mixture was stirred for 2 h. Chromatography gave **29** (1.64 g, 58%) as a purple glass.

** *Spectroscopic data* for **29**: δ_H ($CDCl_3$) −2.75 (2 H, porphyrin 21,23-H_2), 0.89 (2 H, m, $NCH_2CH_2CH_2CH_2CH_2CO$), 1.25–1.65 (6 H, m, $NCH_2CH_2CH_2CH_2CH_2CO$ + Glu β-H_2), 1.9–2.4 (4 H, Glu γ-H_2 + $CH_2CH_2CH_2CO$), 3.0–3.1 (8 H, m, $NCH_2CH_2CH_2$ + 2 × NCH_3), 3.35 (4 H, br, NCH_2CH_2N), 3.85–4.05 (4 H, m, 2 × Sar-H_2), 4.15–4.25 (2 H, m, Gly-H_2), 4.42 (1 H, m, Glu α-H), 5.13 (4 H, br s, 2 × $PhCH_2O$), 7.25–7.33 (14 H, m, 4 × NH + 2 × benzyloxy Ph-H_5), 7.70–7.76 (11 H, m, 3 × porphyrin-Ph 3,4,5-H_3 + porphyrin-C_6H_4N 2,6-H_2), 7.96 (2 H, m, 2 × NH), 8.12 (2 H, d, *J* 8.2 Hz, porphyrin-C_6H_4N 3,5-H_2), 8.17–8.23 (6 H, m, 3 × porphyrin-Ph 2,6-H_2), 8.84 (8 H, br s, porphyrin 2,3,7,8,12,13,17,18-H_8); *m/z* (FAB) 1381.6184 (M + H) ($C_{81}H_{81}N_{12}O_{10}$ requires 1381.6199).

†† Reactions took place at ambient temperature, unless otherwise stated.

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PUBLICATION 68

Unexpected Formation of a 5-Trifluoromethyloxazole
from a 1,2-Dibenzamidoalkene

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Unexpected formation of a 5-trifluoromethyloxazole from a 1,2-dibenzamidoalkene

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Abstract

An unusual cyclisation of *Z*-1,2,4-tris(benzamido)butene with trifluoroacetic anhydride gives *Z*-4-[1,3-bis(benzamido)prop-1-enyl]-2-phenyl-5-trifluoromethyloxazole (**3**). The structure and stereochemistry were confirmed by ^{1}H, ^{13}C and ^{19}F NMR, together with ^{1}H-^{1}H COSY, ^{1}H-^{1}H NOESY and $^{1}H \rightarrow {}^{19}F$ and $^{19}F \rightarrow {}^{1}H$ heteronuclear NOE experiments. Trifluoroacetylation at the less sterically hindered *N*-acyl-enamine position, with subsequent cyclisation and 1,4-elimination is proposed.

Keywords: 5-Trifluoromethyloxazole; 1,2-Dibenzamidoalkene; Cyclisation; NMR spectroscopy; Stereochemistry

1. Introduction

As part of a programme of synthesis of trifluoromethyl-heterocycles for medicinal and pH sensor applications [1,2], we required 2-trifluoromethylhistamine. This compound has been prepared by Bamberger cleavage of histamine (**1**), followed by treatment with boiling trifluoroacetic anhydride and acid-catalysed hydrolysis of the side-chain benzamide. Kimoto et al. [3] also noted the formation of a significant unidentified side-product. We now report the preparation and characterisation of this material as a 5-trifluoromethyl-4-alkenyloxazole which is formed by an interesting alternative cyclisation.

2. Results and discussion

As expected [4], Bamberger fragmentation of histamine (**1**) gave *S*-1,2,4-tris(benzamido)butene (**2**). Treatment with boiling trifluoroacetic anhydride, followed by methanol, gave a poorly soluble solid in moderate yield. The high resolution CI mass spectrum showed a major $(M+H)^{+}$ peak at m/z 492.1535, corresponding to the molecular formula $C_{27}H_{20}F_3N_3O_3$. ^{19}F NMR spectroscopy showed only one singlet at δ −58.89 ppm; thus one CF_3 group or two equivalent CF_3 groups are present. The ^{1}H NMR spectrum revealed the presence of only one CH_2 (δ 4.19 ppm) and one alkene proton, along with 15 aromatic protons and two NH protons. Hence one of the CH_2 groups of the triamide **2** has become involved in the reaction. A ^{1}H-^{1}H COSY spectrum indicated coupling from the CH_2 to the vinylic-H and to the upfield NH (Fig. 1). No coupling was evident between the alkene-H and either NH. These data show compound **3** to have the alkenyloxazole structure shown. A ^{1}H-^{1}H NOESY experiment gave a cross-peak between the downfield NH (δ 10.50 ppm) and the CH_2, showing *Z* stereochemistry about the C=C double bond (Fig. 1). An NOE enhancement was observed in the ^{19}F signal of the CF_3 group on irradiation at the ^{1}H frequency of the alkene-H, but not on irradiation at the ^{1}H frequency of the CH_2, which corroborated this structural assignment (Fig. 1). The converse NOE experiment, involving irradiation at the δ_F of the CF_3 group, showed enhancement of the ^{1}H signals of the downfield NH, the vinylic-H and, more weakly, to the 2,6-H_2 of one benzamide (Fig. 1).

To rationalise this unexpected cyclisation, a mechanism such as that shown in Scheme 1 is proposed. Acylation of the triamide **2** at the more sterically accessible enamine position is followed by nucleophilic attack of the amide oxygen on the trifluoromethyl ketone. Trifluoroacetylation of the tetrahedral intermediate at oxygen provides an excellent leaving group for the 1,4-elimination to afford the alkenyloxazole **3**.

3. Experimental details

NMR spectra were obtained of a solution in $(CD_3)_2SO$ at 70 °C to ensure complete solution of **3**, using JEOL EX-400

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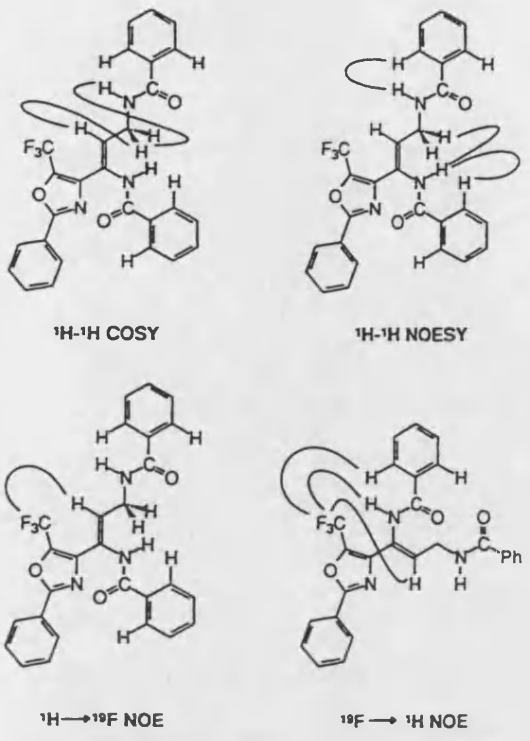
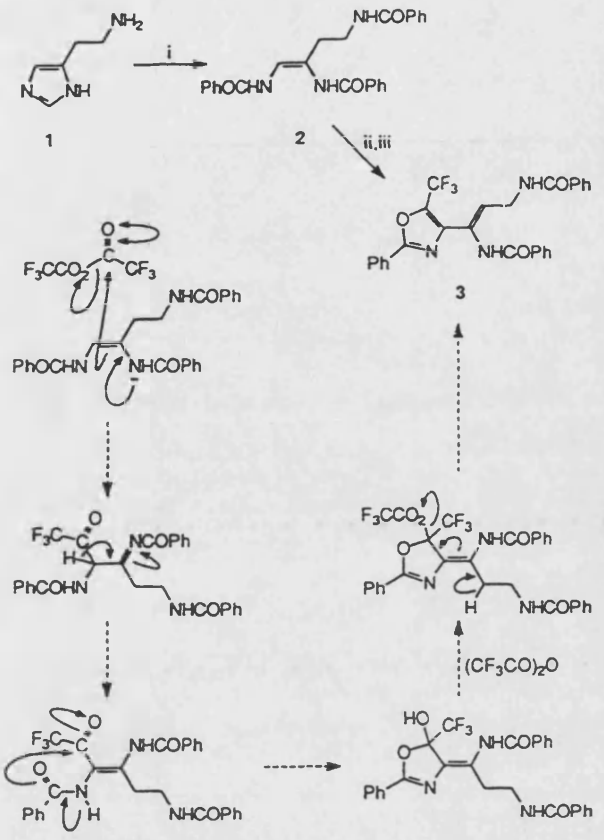


Fig. 1. Principal COSY and NOE interactions for the characterisation of 3. Other interactions were observed between the protons attached to the individual benzene rings.

and Bruker AC-250 instruments. The ^{19}F NMR chemical shift is referenced to $CFCl_3$.

3.1. Z-4-(1,3-Bis(benzamido)prop-1-enyl)-2-phenyl-5-trifluoromethyloxazole (3)

Z-1,2,4-Tris(benzamido)butene (2) [4] (388 mg, 0.91 mmol) was boiled under reflux with trifluoroacetic anhydride (4 ml) for 16 h. The excess reagent was evaporated and the residue was boiled under reflux in methanol (5 ml) for 1 h. The solid was collected by filtration from the cooled mixture to give the oxazole 3 (78 mg, 18%) as a white solid, m.p. 216–217 °C. ^{1}H NMR δ: 4.19 (br t, 2H, J = 5 Hz, CH_2); 6.29 (br t, 1H, J = 5 Hz, C=CH); 7.58–7.68 (m, 9H, 3 × Ar 3,4,5-H_3); 7.99 (d, 2H, J = 6.7 Hz, 2,6-H_2 of oxazole-2-Ph); 8.08–8.13 (m, 4H, 2 × benzamide 2,6-H_2); 9.08 (br t, 1H, N*H*CH_2); 10.50 (s, 1H, NH) ppm. ^{13}C NMR δ: 36.59, 119.07 (q, J_{C-F} = 268 Hz, CF_3); 124.87; 126.00; 126.48; 126.53; 129.90; 127.17; 127.94; 128.11; 128.98; 130.96; 131.38; 131.73; 132.53 (q, J_{C-F} = 44 Hz, C–CF_3); 133.43; 133.83; 140.54; 160.38; 164.96; 166.62 ppm. ^{19}F NMR δ: −58.89



Scheme 1. Synthesis of the trifluoromethyloxazole 3 and proposed mechanism. Reagents: i, PhCOCl; ii, $(CF_3CO)_2O$; iii, MeOH.

(s) ppm. MS (CI) m/z: 492.1535 (M + H) ($C_{27}H_{21}F_3N_3O_3$ requires 492.1535).

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PUBLICATION 69

Inhibition of Nitric Oxide ($NO^{\bullet}$) Production in Murine Macrophages by Flavones

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INHIBITION OF NITRIC OXIDE ($NO^{\cdot}$) PRODUCTION IN MURINE MACROPHAGES BY FLAVONES

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Abstract—The effect of flavone (2-phenylbenzopyran-4-one) and three amino-substituted flavones on the production of nitrite by murine activated peritoneal macrophages was studied *in vitro*. Activated peritoneal macrophages obtained from mice pre-treated with concanavalin A (Con A) (*in vivo*), after exposure *in vitro* to lipopolysaccharide (LPS) at a concentration of 100 ng/ml, produced nitrite (20.3 ± 2.5 nmol/10^6 cells), as measured after 24 hr by the Griess reaction. Stimulation of production of nitrite was inhibited by N^G-monomethyl-L-arginine, suggesting that nitrite was formed via nitric oxide ($NO^{\cdot}$) as a product of metabolism of arginine. Stimulation was inhibited by flavone and the aminoflavones (20–100 μM). 3′-amino-4′-hydroxyflavone was the most potent inhibitor of nitrite production. Genistein (5,7-dihydroxy-3-(4-hydroxy-phenyl)-4H-1-benzopyran-4-one) also inhibited production of nitrite, by a mechanism that appears not to involve protein tyrosine kinases. These results suggest that the flavones can modulate the immune responses and the inflammatory reactions by controlling production of nitric oxide.

Key words: macrophages, nitric oxide, flavones, 3′-amino-4′-hydroxyflavone, $NO^{\cdot}$

Macrophages are known to play an important role in host defence mechanisms. Among a variety of mediators released by activated macrophages [1, 2], nitric oxide ($NO^{\cdot}$) has been identified as a potent molecule that may exert regulatory or cytotoxic effects depending on the concentration acting on the target cell [3, 4]. The inducible form of the arginine-dependent enzyme nitric oxide-synthase generates high, toxic amounts of nitric oxide, which enable the activated macrophages to destroy tumour cells, intracellular bacteria, parasites [5, 6, 7], and even normal tissue in situations of autoimmune reactivity [8, 9]. Earlier studies have shown that functions of activated macrophages such as killing of tumour cells, release of cytokines, and generation of oxygen radicals, can be regulated by flavones (2-phenylbenzopyran-4-ones) [10–12]. Various flavones have been shown to be good scavengers of free radicals [13–18] and to act as natural antioxidants. They have also been shown to inhibit oxido-reductases [19–21], thus preventing the formation of free radicals resulting from the reduction of oxygen. These two mechanisms can account for the role of many flavonoids in protecting cells from oxidative damage.

The present study was designed to investigate the role of flavones in the regulation of $NO^{\cdot}$ release from activated murine macrophages. Nitrite and nitrate are formed as end products of the metabolism of reactive nitrogen intermediates, with the measurement of nitrite using the Griess reagent (with or without the reduction of nitrate) being generally employed as a marker of formation of nitric oxide.

MATERIALS AND METHODS

Mice

Specific pathogen-free 8-to-10 week old BALB/c male mice were purchased from the Institute of Oncology, Gliwice, Poland.

Reagents

Flavone (2-phenyl-4H-1-benzopyran-4-one, sample 1), 4′-aminoflavone (sample 2), and 3′-amino-4′-hydroxyflavone (sample 3) were synthesised as previously described [22]. 3′-amino-4′-methoxyflavone (sample 4) was prepared by reduction of 4′-methoxy-3′-nitroflavone with tin(II) chloride by the general method [22] (^{1}H NMR δ 6.83 (1 H, s, 3-H), 7.22 (1 H, d, J = 8.4 Hz, 5′-H), 7.50 (1 H, dd, J = 8.4, 7.0 Hz, 6-H), 7.69 (1 H, d, J = 8.1 Hz, 8-H), 7.86 (1 H, ddd, J = 8.4, 7.0, 1.8 Hz, 7-H), 7.94 (1 H, dd, J = 8.4, 2.2 Hz, 6′-H), 8.04 (1 H, dd, J = 8.4, 1.8 Hz, 5-H), 8.06 (1 H, d, J = 2.2 Hz, 2′-H), 9.7 (2 H, br, NH_2), 11.7 (1 H, s, OH)). The structures of the flavones used in this work are shown in Fig. 3. Phosphate-buffered saline solution (PBS), RPMI 1640 medium without phenol red, and heat-inactivated foetal calf serum (FCS, low in endotoxin) were purchased from GIBCO Life Technologies Ltd. (Paisley, U.K.). Thioglycollate broth, phenol red solution (0.5% aqueous solution of the sodium salt), N-(1-naphthyl)ethylenediamine dihydrochloride, sulphanilamide, and heparin sodium (pyrogen-free) were purchased from Serva Chemicals (Heidelberg, Germany). Lipopolysaccharide (LPS) from *E. coli* (serotype O127:B8), Concanavalin A (Con A) from *Canavalia ensiformis*, N^G-monomethyl-

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Abbreviations: Con A, concanavalin A from *Canavalia ensiformis*; NMR, nuclear magnetic resonance spectrum; PBS, phosphate-buffered saline solution; FCS, heat-inactivated foetal calf serum; LPS, lipopolysaccharide from *E. coli* (serotype O127:B8); N^GMMA, N^G-monomethyl-L-arginine monoacetate salt.

L-arginine monoacetate salt (N^GMMA), daidzein (7-hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one), and genistein (5,7-dihydroxy-3-(4-hydroxy-phenyl)-4H-1-benzopyran-4-one) were purchased from Calbiochem (La Jolla, CA, U.S.A.). Monoclonal antibody anti-mouse Thy-1,2 fluorescein conjugate was purchased from Becton Dickinson Immunocytochemistry Systems (Mountain View, CA, U.S.A.). Flavones (1–4), isoflavones (daidzein and genistein), LPS, and N^GMMA were dissolved in phenol red-free RPMI 1640 medium, and diluted immediately before use.

Collection and cultivation of mouse peritoneal exudate macrophages

Macrophages were obtained from mice given an i.p. injection of sterile thioglycolate broth (1 ml) 4 d prior to harvest and the solution of Con A (100 μg/ml) in PBS (1 ml) 18 hr prior to harvest. Mice were killed by cervical dislocation, and cells were collected by washing the peritoneum with PBS (5 ml). The macrophage populations were enriched by adherence to plastic in 24-well plates (Falcon, Becton Dickinson, Lincoln Park, NJ, U.S.A.) with calculated 10^6 macrophages per well. Non-adherent cells were removed after 120 min of incubation, and the macrophages were cultured with or without tested agent in RPMI 1640 medium without phenol red, supplemented with 10% heat inactivated FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% CO_2 in air. The adherent population contained >96% macrophages as assessed by May-Grünwald/Giemsa staining and biochemical criteria (nonspecific esterase staining), and <2% T-lymphocytes by fluorescence microscopy after fluorescent antibody labelling with anti Thy-1,2 monoclonal antibody. More than 98% of the cells were viable as determined by exclusion of neutral red [23], also after incubation with all reagents.

The macrophage monolayers were then covered by culture medium (2 ml) with flavones, N^GMMA, daidzein, or genistein (concentration range 1–300 μM) and LPS (100 ng/ml), and incubated for further 6, 18, 24, 48, and 72 hr at the same temperature and atmosphere as above. In all experiments, each concentration of flavone was tested in triplicate. Control macrophages were prepared and cultured in culture medium without added flavones.

Nitrite assay

Nitrite concentrations in the culture medium were measured by a microplate assay method, based on the Griess reaction [24]. Equal volumes of culture medium supernatant and Griess reagent (0.5% sulfanilamide, 0.05% naphthylene-diamide dihydrochloride in 2.5% H_3PO_4) were added to Eppendorf tubes and incubated at 20°C for 10 min. The tubes were then centrifuged for 5 min at 8500 × g. The absorbance of culture medium and Griess reagent at 550 nm was determined with SPECOL. Nitrite content was determined by using sodium nitrite as standard. Data were expressed as nmol nitrite per 10^6 cells originally plated. In all experiments, the nitrite content in wells containing medium without cells was measured and subtracted.

Statistical analysis

For statistical evaluation of the data, the Student's *t*-test was used.

RESULTS

In the presence of LPS, production of nitric oxide was found to be induced in Con A-stimulated macrophages in a time- and dose-dependent manner (Figs. 1 and 2). Con A-stimulated macrophages were obtained from mice injected with thioglycolate broth 4 d previously and with the solution of Con A 18 hr before being killed. Thioglycolate-elicited macrophages released small amounts of nitrite (1.3 ± 0.3 nmol/10^6 cells/24 hr). Elicited macrophages were prepared from mice injected 4 d previously with thioglycolate broth. Con A-stimulated macrophages also released small amounts of nitrite in the absence of LPS (Fig. 1).

Con A-stimulated macrophages produced L-arginine-dependent nitric oxide in the presence of LPS. N^GMMA (300 μM) inhibited nitrite production by 95 ± 3%. N^GMMA (400 μM) had no significant effect on the viability of macrophages exposed to LPS (100 ng/ml). Genistein, an isoflavone that inhibits tyrosine-specific protein kinases, and daidzein, an isoflavone analogue that is inactive against these tyrosine kinases, also significantly decreased the production of nitrite (Table 1).

In the next experiment, the flavones were investigated for their involvement in release of nitric oxide from Con A- and LPS-activated macrophages. Activated macrophages were found to produce detectable nitrite, which ranged in individual experiments from 17.8 to 22.8 nmol/10^6 cells/24 hr. Concentrations of the four synthetic flavones in the range 20–100 μM reduced the production of nitrite (Fig. 3). 3′-amino-4′-hydroxyflavone (3) was the most potent inhibitor, with significant inhibition at 1 μM and 84 ± 2% inhibition at 20 μM. Similar results were obtained following culture for 48 hr in the presence of the agents. Thomsen *et al.* [25] have pointed out that nitric oxide is converted both to nitrite and to nitrate. However, in representative control experiments in which the nitrate was reduced to nitrite with *Aspergillus* nitrate reductase and NADPH prior to assay, nitrate was found present in only trace amounts (results not shown). Thus, in our system, production of nitrite is a good measure of production of nitric oxide.

The viability of the macrophages was >92% following

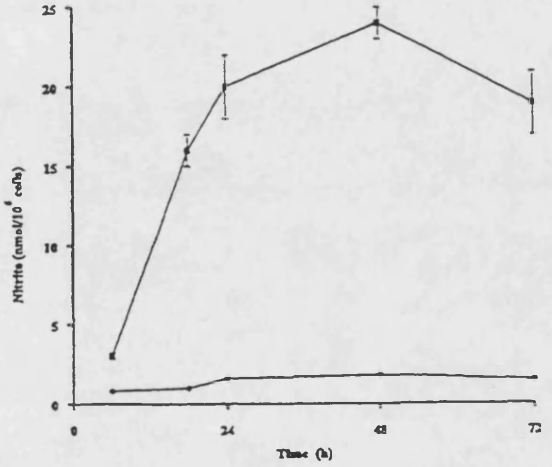

Fig. 1. Amounts of nitrite produced by macrophages cultured for 6, 18, 24, 48, or 72 hr without LPS (●) and with LPS (100 ng/ml) (■). Results are representative for three experiments.

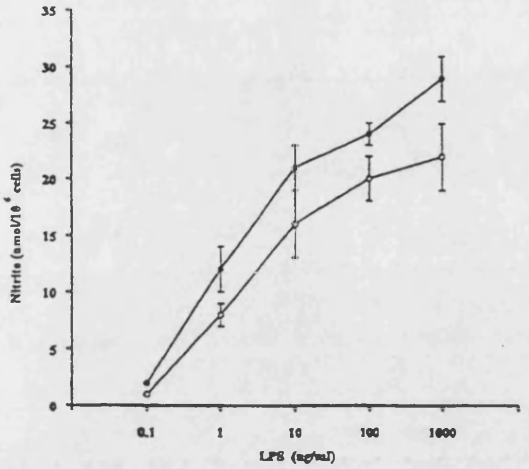

Fig. 2. Dose-dependent effects of LPS *in vitro* or production of nitrite by macrophages. Macrophages were incubated for 24 hr (○) or 48 h (●) with increasing concentrations of LPS. Each value is the mean ± *SD* of three experiments.

Table 1. Inhibitory effects of the isoflavones, genistein, and daidzein, and of N^GMMA on the nitrite produced by macrophages

Compound	Inhibition [%]
Genistein (50 μM)	48 ± 3
Daidzein (50 μM)	94 ± 2
N^GMMA (300 μM)	95 ± 3

Macrophages were incubated for 24 hr with LPS (100 ng/ml) in the presence of the inhibitors. Data show average ± *SD* from three independent experiments performed in triplicate.

treatment with agents (flavones and isoflavones) at concentrations up to 100 μM and >94% with no treatment. Hence, inhibition of production of nitrite is unlikely to be due to toxicity of the compounds. Additionally, the flavones and isoflavones had no quenching effect on the Griess reagent at the concentration used.

DISCUSSION

The adherent populations from mouse peritoneal cavities were used as macrophage models in this study. Thioglycollate-elicited peritoneal macrophages from BALB/c mice released significant amounts of nitrite upon stimulation with Con A (*in vivo*) and LPS (*in vitro*). This model for activation of macrophages has been reported [26, 27]. These activated macrophages produce a variety of biologically active molecules both in their normal role and in pathological immune responses and inflammatory reactions. Thioglycollate-elicited and Con A + LPS-primed macrophages express cytostatic activity against P815 tumour cells, and release interleukin-6 and nitrite [26]. Similarly activated macrophages secrete reactive oxygen intermediates (Król W, Czuba ZP and Threadgill MD, unpublished results).

We have shown here that flavone and three amino derivatives inhibit production of nitrite, a chemical product of nitric oxide. The cellular mechanisms of the effect are not clear, but may be related to known biological effects of the flavones, such as antioxidant properties and inhibition of cellular enzymes involved in signal transduction. Some phenolic compounds are known to have radical scavenging activities, and many isoflavonoids and related compounds reportedly possess strong antioxidative activities [28, 29]. However, direct reaction of nitric oxide or nitrite with the flavones, leading to apparent inhibition of production of $NO^{\cdot}$, is rendered highly unlikely by consideration of the stoichiometry of the experiments involving the most potent flavone (3). At 10 μM, the experimental volume (2 ml) contains 20 nmol of 3, yet the loss of production of nitrite compared to control is 16 nmol; at 1 μM, the experimental volume contains 2 nmol of 3, yet the loss of production of nitrite is approximately 4 nmol. These data preclude stoichiometric reaction of 3 with nitric oxide or nitrite from being any more than a very minor contribution to the inhibition observed.

Prior to this work, the only true flavone to have been shown to influence production of nitric oxide by macrophages was flavone-8-acetic acid [25]. This 8-substituted flavone has stimulatory activity, in contrast to the inhibitory activity demonstrated here for the unsubstituted and 3′,4′-substituted flavones 1–4. Indeed, it has recently been suggested that decarboxylation of flavone-8-acetic acid by the radical nitrogen dioxide may contribute to its apparent antitumour activity [30].

Three specific protein tyrosine kinase inhibitors (genistein, tyrphostin, and herbimycin A) have been reported to block production of nitrite in both C3H/HeN and C3H/HeJ macrophages [31]. Flavone itself and several aminoflavones are inhibitors of protein tyrosine kinase activity *in vitro* [22, 32, 33], with a variety of potencies. Cushman *et al.* reported that a series of 4′-aminoflavones inhibit the activity of the protein tyrosine kinase $p56^{lck}$; the most potent, 4′-amino-6-hydroxyflavone, inhibits this activity with IC_{50} = 1.2 μM [33]. In our previous study on the activity of a series of synthetic flavones against protein tyrosine kinases [22], we identified 3′-amino-4′-methoxy-flavone (4) as the most potent inhibitor of the EGF receptor tyrosine kinase activity derived from A431 cells (42% inhibition at 50 μM). This compound was inactive against the corresponding activity of p*tabl*50 at 500 μM, whereas quercetin (3,3′,4′,5,7-pentahydroxyflavone) had K_i = 3.7 μM. Interestingly, compound 4 showed the greatest selective cytotoxicity towards ANN-1 cells, which are Abelson-transformed 3T3 cells. In the present study, the isoflavone genistein was also found to inhibit the production of nitrite by macrophages. However, the analogous isoflavone daidzein, which does not inhibit protein tyrosine kinases, was found to be a more potent inhibitor of production of nitric oxide by these macrophages than is genistein. This suggests that inhibition of tyrosine kinases may not be involved in the mechanism of inhibition of release of nitric oxide in these macrophages.

In a subsequent study, a related series of flavones were evaluated for inhibition of the generation of reactive oxygen species by macrophages that had been primed by LPS-polyinosinic-polycytidylic acid, as shown by inhibition of luminol-dependent chemiluminescence [12]. Of these flavones, 3′-amino-4′-hydroxyflavone (3) was the most potent inhibitor, causing 93% inhibition at 100 μM, 52% inhibition at 10 μM, and 36% inhibition at 1.0 μM, corresponding to IC_{50} = 3.3 μM.

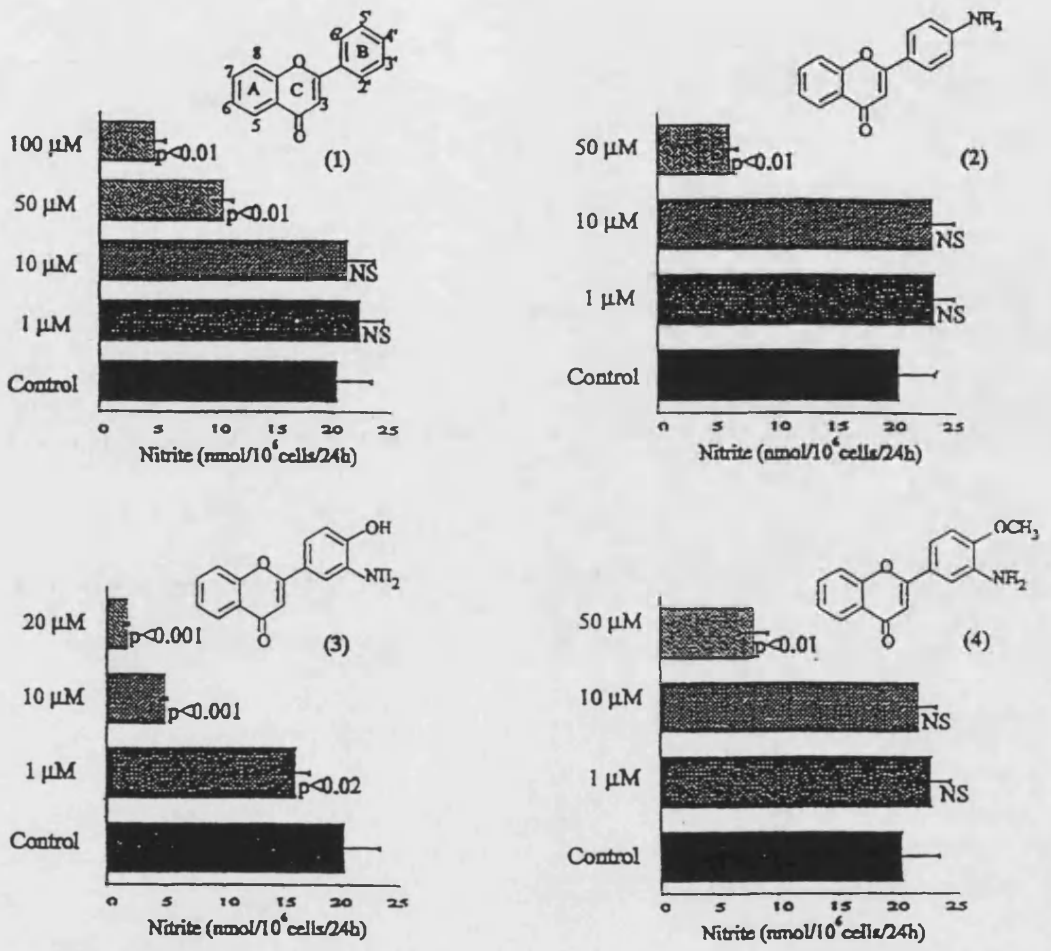

Fig. 3. Effects of flavone (1), 4′-aminoflavone (2), 3′-amino-4′-hydroxyflavone (3), and 3′-amino-4′-methoxyflavone (4) on production of nitrite by macrophages. Macrophages were incubated with LPS (100 ng/ml), without flavones (control) or with flavones, for 24 hr. Concentrations of nitrite (nmol/10^6 cells, mean ± *SD* of three wells from three independent experiments) produced by control macrophages were 20.3 ± 2.5.

Flavone (1), 4′-aminoflavone (2), and 3′-amino-4′-methoxyflavone (4) were less potent, causing 55%, 70%, and 65% inhibition, respectively, at 100 μM, and insignificant inhibition at 10 μM and at 1.0 μM. Interestingly, the concentrations of these four flavones required for inhibition of release of nitric oxide by the macrophages closely match these values (Fig. 3). These data strongly suggest that production of nitric oxide is a major controlling factor in generation of reactive oxygen species upon activation of the macrophages. Thus, flavones may have a role to play in modulating inflammatory and immune responses by affecting production of nitric oxide. Studies as to whether the flavones act as direct inhibitors of nitric oxide synthase or act upon a control system are in progress, and will be reported separately.

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LABELLED COMPOUNDS OF INTEREST AS ANTITUMOUR AGENTS - V[1]. SYNTHESES OF [^{18}O]-5-METHYLISOQUINOLINONE AND 1-(FURAN-2-YL-[^{18}O]-METHOXY)-5-METHYLISOQUINOLINE

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SUMMARY

Treatment of 2-methylcinnamic acid with $H_2{}^{18}O$ at 100°C under acidic conditions leads to high incorporation of ^{18}O by exchange. Methods have been developed for chemically and isotopically efficient conversion to the corresponding [carbonyl-^{18}O] methyl ester, to [^{18}O]-5-methylisoquinolinone (an inhibitor of DNA repair) and to 1-(furan-2-yl-[^{18}O]-methoxy)-5-methylisoquinoline.

Introduction

The enzyme poly(ADP-ribose)polymerase (PARP, EC 2.4.2.30) catalyses the transfer of ADP-ribose units from NAD^+ to form a polyanionic polymer on histones and other acceptor proteins near the site of damage to DNA[2-4]. Inhibition of PARP leads to inhibition of the processes of repair of damaged sites in DNA[5-7] and thus to potentiation of the antitumour effects of many forms of radiotherapy[8-11] and chemotherapy[12-14]. Potent inhibitors of PARP include benzamides and analogues in which the conformation of the amide is constrained relative to the aromatic ring, by incorporation into a lactam[8,15,16] or by hydrogen-bonding[16]. Of these lactams, 5-substituted isoquinolinones have been shown[8,15] to be among the inhibitors with the greatest potency. During our studies on drugs which sensitise tumours to the cytotoxic effects of radiotherapy and chemotherapy[17], we required a 5-substituted isoquinolinone PARP inhibitor labelled with ^{18}O at the carbonyl oxygen. In this paper, we report our development of syntheses of methyl [carbonyl-^{18}O]-*E*-3-(2-methylphenyl)propenoate **4**, a model intermediate to check satisfactory isotopic incorporation, of [^{18}O]-5-methylisoquinolinone **7** and of a potential pro-drug derivative, 1-(furan-2-yl-[^{18}O]-methoxy)-5-methylisoquinoline **10**.

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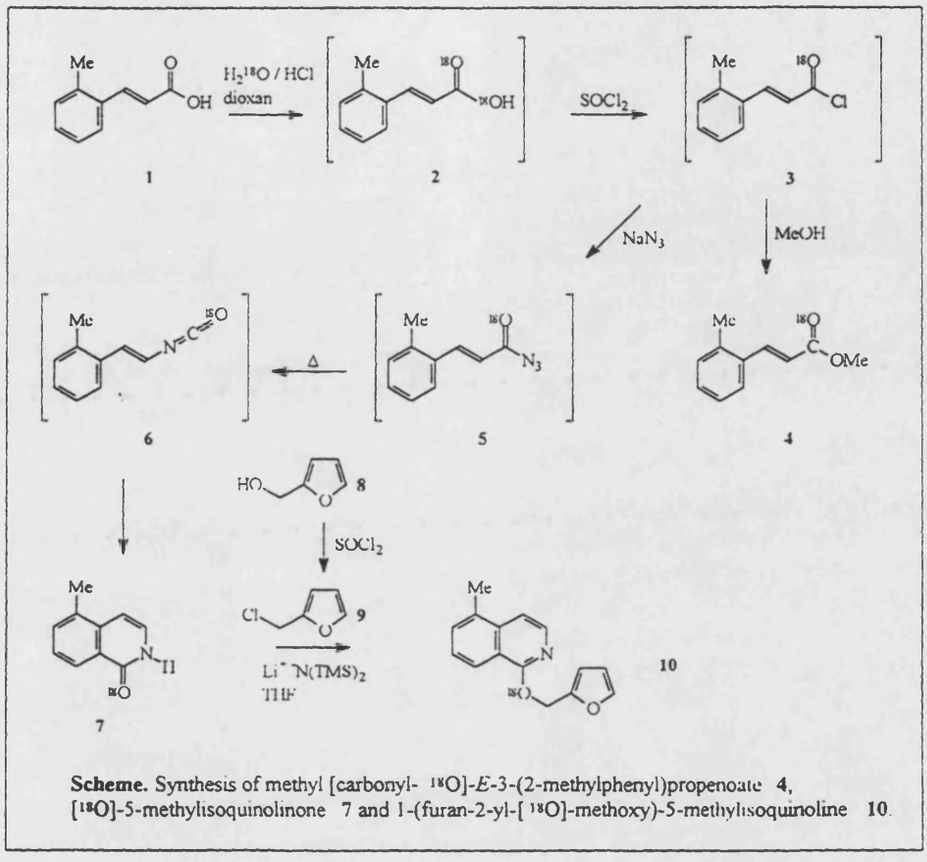
Results & Discussion

A convenient synthesis of isoquinolinones by Curtius rearrangement of cinnamyl azides and thermal cyclisation, in a one-pot process, has been described by Eloy and Deryckere[18]. The Curtius reaction involves a true rearrangement of the intermediate acyl nitrene to form the isocyanate. Thus ^{18}O present in the carbonyl group should be retained in the isocyanate. The subsequent mechanistic steps, thermal isomerisation of the C=C and cyclisation, also take place under conditions where the oxygen atom should be retained in the isoquinolinone. The synthetic route, therefore, requires the ^{18}O-labelled 2-methylcinnamyl azide **5** as a starting material for the synthesis of the ^{18}O-labelled 5-methylisoquinolinone **7**.

We considered that **5**, in turn could be derived from the corresponding ^{18}O-labelled acid chloride **3** and the ^{18}O-labelled carboxylic acid **2**. An initial series of model experiments was devised to develop a procedure for incorporation of ^{18}O into 2-methylcinnamic acid **1**, forming **2**, and for conversion to the acid chloride **3**, under conditions which would not permit loss of ^{18}O by exchange. ^{18}O was incorporated by heating the carboxylic acid **1** in 1,4-dioxan with twenty equivalents of $H_2{}^{18}O$ under acidic conditions to promote exchange. The solvent and excess water were removed by distillation in a closed system to give the isotopomer **2**. Since this acid is capable of losing ^{18}O by exchange with atmospheric water, it was rapidly converted to the corresponding acid chloride **3** with thionyl chloride. To check the level of incorporation of ^{18}O, the acid chloride was quenched with methanol. Mass spectrometry showed the ester **4** to contain 8.0 ± 0.7% ^{18}O in the carbonyl group, confirming that the exchange process had proceeded satisfactorily and that little ^{18}O had been lost during the subsequent reactions.

Now that the initial exchange reaction had been developed and conversion to the acid chloride **3** had been established, attention was focussed on the main isoquinolinone-forming process. The ^{18}O-labelled acid chloride **3** was prepared as before but on a multimillimole scale The acid chloride **3** should not be subject to exchange of ^{18}O under aqueous conditions without hydrolysis, so conversion to the acyl azide **4** was effected by treatment with sodium azide in aqueous acetone. Any material which may have been hydrolysed to the acid would not be converted to the acyl azide by this process. The Curtius reaction was carried out in boiling diphenyl ether at *ca.* 260°C, forming the isocyanate **6**, which was isomerised $E \rightarrow Z$ and cyclised under the same conditions, giving the ^{18}O-labelled 5-methylisoquinolinone **7** in good yield. Again, the isotopic composition was determined by mass spectrometry; **7** was shown to contain 8.0% ^{18}O at the carbonyl oxygen. This represents the same incorporation as was seen for the methyl 2-methylcinnamate **4**, indicating no loss of ^{18}O in steps after the acid chloride.



Scheme. Synthesis of methyl [carbonyl- ^{18}O]-*E*-3-(2-methylphenyl)propenoate **4**, [^{18}O]-5-methylisoquinolinone **7** and 1-(furan-2-yl-[^{18}O]-methoxy)-5-methylisoquinoline **10**.

For the synthesis of the *O*-furanylmethyl derivative **10**, two synthetic approaches were possible; (*i*) reaction of furan-2-[^{18}O]-methanol, as its alkoxide, with 1-chloro-5-methylisoquinoline or (*ii*) alkylation of the anion of the isoquinolinone **7** with an electrophilic furanylmethyl compound. The former was discounted, as this would require development of a synthesis of a new ^{18}O-labelled intermediate, the furanmethanol. Nevertheless, the reaction of unlabelled sodium furanmethoxide with 1-chloroisoquinoline was investigated but was found to lead only to destruction of the furan at the high temperature of the reaction. The second approach also presented some problems, as furanylmethyl electrophiles are notoriously unstable.

2-Chloromethylfuran **9** was prepared by treatment of the corresponding alcohol **8** with thionyl chloride by the general method of Tarrago *et al*[19]. This unstable material was allowed to react with the lithium anion of the ^{18}O-labelled 5-methylisoquinolinone **7** at reflux in tetrahydrofuran. To counteract the loss of **8** by decomposition during this alkylation, it was used in large excess. The ^{18}O-labelled furanylmethoxyisoquinoline **10** was obtained in good chemical yield and with excellent

isotopic enrichment (8.0% ^{18}O). The equivalence of the isotopic enrichment of 7 and 10 demonstrates that no loss of ^{18}O has occurred during the alkylation.

Conclusion

Efficient techniques have been developed for the incorporation of ^{18}O into a *Ar*-substituted cinnamic acid by exchange with $H_2{}^{18}O$ and for subsequent conversion to the carbonyl-^{18}O-labelled isoquinolinone 7 and the furanylmethyl ether 10. These methods should be applicable to syntheses of other ^{18}O-labelled cinnamate esters and to other isoquinolinones which do not bear strong electron-withdrawing groups. The results of biological and biomimetic studies with 7 and 10 will be reported elsewhere.

Experimental

Jeol GX270 and EX400 instruments furnished the NMR spectra of solutions in $CDCl_3$; the internal standard was tetramethylsilane. Melting points are uncorrected. Solvents were evaporated under reduced pressure. The chromatographic stationary phase was silica gel. Brine refers to a saturated solution of sodium chloride in water. [^{18}O]-Water (10 atom %) was obtained from the Aldrich Chemical Company.

Methyl [carbonyl-^{18}O]-*E*-3-(2-methyl)phenylpropenoate (4). *E*-3-(2-methyl)phenylpropenoic acid 1 (53 mg, 330 μmol) was boiled under reflux with [^{18}O]-water (120 μl, 10 atom %) and hydrogen chloride (1.0 M in diethyl ether, 150 μl) in dry 1,4-dioxan (1.5 ml) for 24 h. The solvents and excess reagent were distilled off under nitrogen and the residue was stirred with thionyl chloride (3.0 ml) and dimethylformamide (10 μl) for 16 h. The excess reagent was evaporated. Methanol (2.0 ml) was added and the mixture was stirred for 1 h. Chromatography (ethyl acetate) yielded 4 (46 mg, 79%) as a pale yellow oil (lit.[20] unlabelled compound is an oil): δ_H 2.43 (3 H, s, Ar-Me), 3.81 (3 H, s, OMe), 6.36 (1 H, d, J = 15.9 Hz, HC=C), 7.18-7.28 (3 H, m, Ar 3,4,5-H_3), 7.54 (1 H, d, J = 7.0 Hz, Ar 6-H), 8.00 (1 H, d, J = 15.9 Hz, C=CH); δ_C 19.74, 51.62, 118.8, 126.29, 126.35, 129.97, 130.73, 133.32, 137.59, 142.49, 167.40; *m/z* (EI) 178 (2.7%) (M), 176 (33%) (M), 163 (2.0%) (M - Me), 161 (23%) (M - Me), 147 (9%) (M - MeO), 145 (90%) (M - MeO).

[^{18}O]-5-Methylisoquinolinone (7). *E*-3-(2-methyl)phenylpropenoic acid 1 (450 mg, 2.8 mmol) was boiled under reflux with [^{18}O]-water (1.00 ml, 10 atom %) and hydrogen chloride (1.0 M in diethyl ether, 1.5 ml) in dry 1,4-dioxan (15 ml) for 12 h. The solvents and excess reagent were distilled off under nitrogen and the residue was stirred with thionyl chloride (5 ml) and dimethylformamide

(10 μl) for 1.5 h. The excess reagent was evaporated. The acid chloride **3**, in acetone (5 ml), was added to sodium azide (630 mg, 9.7 mmol) in water (2 ml) and acetone (1 ml) at 0°C. The mixture was stirred for 30 min at this temperature. The acyl azide **5** was extracted with dichloromethane and was washed with brine and was dried ($MgSO_4$ / $CaCl_2$). Diphenyl ether (3 ml) was added and the dichloromethane was evaporated at ambient temperature. The residue was added to boiling diphenyl ether (10 ml) during 10 min and the solution was boiled under reflux for 2 h. Evaporation and chromatography (ethyl acetate / hexane 1:1) gave **7** (270 mg, 61%) as a pale yellow solid: mp 178-180°C (lit.[18] mp 184-185°C for the unlabelled compound); δ_H 2.55 (3 H, s, Me), 6.71 (1 H, d, J = 7.3 Hz, 4-H), 7.25 (d, J = 6.4 Hz, 3-H), 7.40 (1 H, dd, J = 7.9, 7.3 Hz, 7-H), 7.52 (1 H, d, J = 7.0 Hz, 6-H), 8.30 (1 H, d, J = 7.9 Hz, 8-H); δ_C 19.17, 103.47, 125.18, 126 10, 126.39, 127.47, 133.45, 137.18, 164.90 (one C_q was not observed); *m/z* (EI) 161.0726 ($C_{10}H_9N^{18}O$ requires 161.0727) (8.7%) (M), 159.0682 ($C_{10}H_9N^{16}O$ requires 159.0684) (100%) (M)

1-(Furan-2-yl-[^{18}O]-methoxy)-5-methylisoquinoline (10). To furan-2-methanol **8** (1.00 g, 10 mmol) in chloroform (10 ml) was added pyridine (1.5 ml). The solution was cooled to -10°C and thionyl chloride (2.0 ml) in chloroform (20 ml) was added. The mixture was stirred at this temperature under nitrogen for 3 h. Hydrochloric acid (10%, 10 ml, 0°C) was added. The organic phase was washed with hydrochloric acid (10%, 0°C) and with aqueous sodium hydroxide (3%, 0°C). The solution was dried ($MgSO_4$ / K_2CO_3) and the solvent was evaporated at ambient temperature to give **9** (1.07 g, 89%) as an unstable orange liquid. The [^{18}O]-isoquinolinone **7** (250 mg, 1.6 mmol), in tetrahydrofuran (25 ml), was treated with lithium hexamethyldisilazide (1.0 M in tetrahydrofuran, 2.0 ml) and the mixture was stirred at ambient temperature for 1 h. 2-Chloromethylfuran **9** (1.00 g, 8.6 mmol), in tetrahydrofuran (25 ml) was added dropwise during 1 h at 0°C, followed by sodium iodide (20 mg). The mixture was boiled under reflux for 18 h. The evaporation residue, in ethyl acetate, was washed with water and with brine and was dried ($MgSO_4$). Evaporation and chromatography (ethyl acetate / hexane 1:5) gave **10** (224 mg, 60%) as a pale yellow oil which crystallised on standing: mp 84-86°C; δ_H 2.51 (3 H, s, Me), 5.19 (2 H, s, CH_2), 6.33 (1 H, dd, J = 3.1, 1.9 Hz, furan 4-H), 6.42 (1 H, d, J = 3.3 Hz, furan 3-H), 6.61 (1 H, d, J = 7.7 Hz, isoquinoline 4-H), 7.21 (1 H, d, J = 7.7 Hz, isoquinoline 3-H), 7.36 (2 H, m, isoquinoline 7-H + furan 5-H), 7.46 (1 H, d, J = 7.2 Hz, isoquinoline 6-H), 8.32 (1 H, d, J = 8.1 Hz, isoquinoline 8-H); δ_C 18.93, 44.33, 103.02, 109.42, 110.64, 110.83, 125.97, 126.55, 130.54, 133.08, 133.17, 135.90, 142.77, 149.78, 162.1; *m/z* (EI) 241.0990 ($C_{15}H_{13}N^{16}O^{18}O$ requires 241.0989) (3.6%) (M), 239.0943 ($C_{15}H_{13}N^{16}O_2$ requires 239.0946) (40%) (M), 81 (100%) (furan-CH_2).

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PUBLICATION 70

3',4'-Aminoflawony Jako Potencjalne Biomodulatory

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3', 4'-aminoflawony jako potencjalne biomodulatory

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Pochodne flawonu (*2-fenylo-4H-1-benzopiran-4-onu*) są interesującą grupą związków o potencjalnych właściwościach przeciwzapalnych i immunotropowych. Niektóre z nich są w trakcie badań klinicznych w immunoterapii nowotworów. Zsyntezowano szereg 3',4'-aminoflawonów inhibitorów kinazy białkowo-tyrozynowej.

Celem przeprowadzonych badań było określenie działania 3',4'-aminoflawonów na sekrecję niektórych produktów przez aktywne makrofagi. Komórki te uzyskiwano z płynu otrzewnowego myszy szczepu BALB/c po stymulacji ConA, makrofagi stymulowano LPS-em. Oznaczano chemiluminescencję makrofagów oraz produkcję tlenku azotu ($NO^{\bullet}$) (pomiar stężenia anionów NO_2^-/NO_3^-) i czynnika martwicy nowotworu (TNF-α). Spośród badanych związków 3'-amino-4'-hydroksyflawon silnie hamował chemiluminescencję i produkcję $NO^{\bullet}$, nie wpływał na sekrecję TNF-α. Natomiast 4'-aminoflawony wykazały stymulujący efekt w zakresie produkcji TNF-α przez makrofagi.

Uzyskane wyniki stanowią zachętę do podjęcia badań na modelach *in vivo*.

PUBLICATION 71

Substituted Flavones as Aryl Hydrocarbon (Ah) Receptor Agonists and Antagonists

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Substituted Flavones as Aryl Hydrocarbon (Ah) Receptor Agonists and Antagonists

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ABSTRACT. The structure-dependent aryl hydrocarbon (Ah) receptor agonist and antagonist activities of the following substituted flavones were investigated: flavone, 4′-methoxy-, 4′-amino-, 4′-chloro-, 4′-bromo-, 4′-nitro-, 4′-chloro-3′-nitro-, 3′-amino-4′-hydroxy-, 3′,4′-dichloro-, and 4′-iodoflavone. The halogenated flavones exhibited competitive Ah receptor binding affinities (IC_{50} = 0.79 to 2.28 nM) that were comparable to that observed for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (1.78 nM). The compounds also induced transformation of the rat cytosolic Ah receptor and induced *CYP1A1* gene expression in MCF-7 human breast cancer cells. However, despite the high Ah receptor binding affinities for these responses, the halogenated flavones were >1000 times less active than TCDD for the other responses. Moreover, for other substituted flavones, there was no correlation between Ah receptor binding affinities and their activities as Ah receptor agonists. For example, 4′-aminoflavone induced CYP1A1 mRNA levels in MCF-7 cells but exhibited relatively low Ah receptor binding affinity (IC_{50} = 362 nM) and did not induce transformation of the rat cytosolic Ah receptor. All of the substituted flavones inhibited TCDD-induced transformation of the Ah receptor, and 4′-iodoflavone, an Ah receptor agonist at high concentrations (1–50 μM), inhibited the transformation at concentrations as low as 0.05 and 0.5 μM. Subsequent interaction studies with TCDD and 4′-iodoflavone confirmed that the latter compound inhibits induction of *CYP1A1* gene expression by TCDD in MCF-7 cells. The results obtained for the substituted flavones suggest that within this structural class of compounds, various substituent groups can affect markedly the activity of each individual congener as an Ah receptor agonist or antagonist. These substituent-dependent differences in activity may be related to ligand-induced conformational changes in the Ah receptor complex and/or support the proposed existence of more than one form of the Ah receptor. BIOCHEM PHARMACOL 51;8:1077–1087, 1996.

KEY WORDS. flavonoids; Ah receptor binding; *CYP1A1* induction/inhibition

TCDD[1] is an industrial and combustion by-product that has been widely identified as an environmental contaminant. TCDD elicits a diverse spectrum of biochemical and toxic responses and is used as a prototype for investigating the toxicology and mechanism of action of structurally related halogenated aromatic compounds [1–4]. Poland and coworkers [5] first demonstrated that [^{3}H]TCDD bound saturably and with high affinity to an hepatic cytosolic protein from C57BL/6 mice, and this protein, named the Ah receptor, has since been widely identified in animal and human tissues [6, 7]. The results of photoaffinity labeling studies indicated that there was considerable intraspecies variability in the apparent molecular weight of the Ah receptor [8], and this is consistent with results of experiments that have cloned the Ah receptor from various species [9–13]. The proposed mechanism of action of TCDD and related compounds was initially derived from studies on induction of *CYP1A1* gene expression by TCDD, 3-methylcholanthrene, and related compounds [3]. The unbound cytosolic Ah receptor is associated with heat shock protein 90; after binding with TCDD, the 9S (270- to 300-kDa) cytosolic Ah receptor complex undergoes a transformation step to form a 5- to 7S (180- to 210-kDa) heterodimeric complex that rapidly accumulates in the nuclear fraction of target cells [14, 15]. The nuclear Ah receptor complex acts as a ligand-induced transcription factor that interacts with 5′-dioxin or xenobiotic response elements (DREs, XREs), and this can result in transactivation of the *CYP1A1* gene [16, 17]. The nuclear heterodimeric complex consists of the Ah receptor and Ah receptor nuclear translocator (Arnt) protein [18], and there is strong evidence that formation of this

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[1] *Abbreviations:* Ah, aryl hydrocarbon; αNF, αnaphthoflavone; DRE, dioxin-responsive element; EROD, ethoxyresorufin O-deethylase; HAP, hydroxylapatite; MCDF, 6-methyl-1,3,8-trichlorodibenzofuran; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; and TCDF, 2,3,7,8-tetrachlorodibenzofuran.

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unique ligand-induced dimeric protein complex plays a major role in modulating expression of several genes [19].

In vitro studies with TCDD and related halogenated aromatics show that there is a rank order correlation between their Ah receptor binding affinities and biochemical/toxic potencies [2, 20, 21]. Moreover, for TCDD and related halogenated aromatic compounds, there is also a correlation between their Ah receptor binding affinities and their activity to induce transformation (i.e. 9–10S to 5–7S) of the rat hepatic cytosolic receptor [22]. The correlations observed for halogenated aromatics have not been investigated extensively within other structural classes of compounds that bind to the Ah receptor. For example, 3-methylcholanthrene binds with high affinity to the Ah receptor but is a relatively weak Ah receptor agonist for several responses [23]. This disparity between binding affinity versus potency as an Ah receptor agonist can be explained, only in part, by the rapid metabolism of 3-methylcholanthrene [24, 25]. However, in ligand-induced cytosolic Ah receptor transformation experiments, 3-methylcholanthrene is significantly less active than TCDD, despite their comparable Ah receptor binding affinities [24].

3′-Methoxy-4′-nitroflavone also binds with high affinity to the rat cytosolic Ah receptor (IC_{50} = 2.27 nM) but does not induce significantly CYP1A1 mRNA levels in MCF-7 human breast cancer cells nor does it induce *in vitro* transformation of the rat hepatic cytosolic Ah receptor [26]. 3′-Methoxy-4′-nitroflavone is an Ah receptor antagonist that inhibited formation of the nuclear Ah receptor complex and induction of *CYP1A1* gene expression by TCDD in MCF-7 cells. This study further investigates the structure-dependent activities of 4′- and 3′,4′-substituted flavones as Ah receptor agonists/antagonists in MCF-7 cells. The haloflavones bound with high affinity to the cytosolic Ah receptor (0.79 to 2.28 nM) but were >1000-fold less active than TCDD as inducers of *CYP1A1* gene expression. Moreover, 4′-iodoflavone was a partial antagonist. There were no apparent structure–activity relationships for the other substituted flavones.

MATERIALS AND METHODS

Chemicals and Biochemicals

The 3′,4′-substituted flavones were synthesized and were >98% pure as determined by spectroscopic measurements and HPLC as described previously [27]. TCDD, [^{3}H]TCDD, ethoxyresorufin, and TCDF were synthesized in this laboratory to >98% purity. MCF-7 human breast cancer cells were obtained originally from the American Type Culture Collection (ATCC, Rockville, MD). All other chemicals and biochemicals were of the highest purity available from commercial sources.

Preparation of Rat Hepatic Cytosol

Liver cytosol from Long-Evans rats was prepared as previously described [26]. Livers were perfused *in situ* with ice-cold HEGD buffer [25 mM HEPES, 1.5 mM EDTA, 1.0 mM dithiothreitol, 10% glycerol (v/v), pH 7.8], homogenized in HEGD (5 mL/g tissue), and centrifuged at 10,000 *g* for 20 min (2°). The resulting supernatant was centrifuged at 105,000 *g* for 1 hr (2°), and the supernatant was stored at −80°. Protein concentrations were determined by the method of Bradford [28].

Cytosolic Receptor Binding Assay

The IC_{50} = and K_i values for competitive receptor binding affinities were determined using rat hepatic cytosol (2 mg protein/mL) and the hydroxylapatite assay procedure essentially as described [29]. Each sample was incubated with [^{3}H]TCDD and the desired competitor at 20° for 2 hr. After incubation, 200 μL of incubate was added to fresh tubes containing HAP slurry (35 mL HAP/100 mL HEGD buffer). Tubes were allowed to incubate for 30 min and centrifuged at 100 *g* for 5 min (4°). The resulting pellet was washed three times with ice-cold HEGD containing 1% Triton X-100. The final pellet was then rinsed with 1 mL ethanol, and the radioactivity was determined by liquid scintillation counting. Non-specific binding was obtained by incubating [^{3}H]TCDD with a 200-fold excess of TCDF. Different concentrations of the ligands were used to determine displacement curves; the IC_{50} = values were defined as the concentrations required to displace 50% of [^{3}H]TCDD and were determined from a log-logit plot of the percentage of [^{3}H]TCDD bound versus log concentrations of the ligands. The results are expressed as means ± SEM for at least 4 separate determinations. K_i values were determined by the method of Cheng and Prusoff [30], as described previously [26].

Ligand-Induced Transformation Assay

Polyacrylamide gel electrophoresis purified complementary strands of the synthetic oligonucleotide, containing a 26-mer consensus DRE sequence (5′-GATCTGGCTCTTCT-CACGCAACTCCG-3′) that corresponds to the DNA sequence from -1997 to -1978 of the mouse *CYP1A1* gene [16], was obtained from Genosys Biotechnologies, Inc. (The Woodlands, TX). The oligonucleotide was labeled at the 5′-end using T4-polynucleotide kinase and [γ-^{32}P]ATP. DNA binding was measured using a gel mobility shift assay [16]. Rat hepatic cytosol (16 mg/mL protein) was incubated for 2 hr at 20° with 5 nM TCDD, 50 μM flavone or 5 nM TCDD plus different concentrations of the flavones; the final concentration of DMSO was 0.2% (v/v). Cytosol (80 μg) was incubated in HEGDK (HEDG + 0.4 M potassium chloride) with 400 ng of poly[d(I-C)] in HEGD buffer and incubated for 15 min at 20°. Following the addition of a ^{32}P-labeled DRE oligonucleotide (0.2 to 1.0 ng; 100,000 cpm), the mixture was incubated for an additional 15 min at 20°. Excess unlabeled DRE (100-fold) was added 5 min prior to addition of ^{32}P-labeled DRE to compete for the specific DNA–protein binding. Protein–DNA complexes

were resolved on a 5–6% polyacrylamide gel (acrylamide: bisacrylamide; 30:0.8) and electrophoresed at 120 V for approximately 3 hr in 0.9 M Tris borate and 2 mM EDTA, pH 8.0. Gels were dried, and protein–DNA complexes were detected using a Betagen Betascope 603 blot analyzer and visualized by autoradiography. The amount of ^{32}P-labeled DRE bound in the ligand-inducible complex was determined by measuring the radioactivity of the specifically bound retarded band and subtracting the amount of radioactivity present in the same position in a non-ligand-treated lane. The difference in radioactivity between these samples represents the ligand-inducible specific binding of the Ah receptor complex to the [^{32}P]DRE. The levels of DNA-binding activity for the various treatment groups were quantified as a percentage of maximal response observed for the TCDD-treated cytosol. The IC_{50} values were obtained from a log-logit plot of the percentage of maximal response obtained for each ligand versus ligand concentration.

Inhibition of Microsomal EROD Activity by Substituted Flavones

Male Long-Evans rats were treated with TCDD (5 μg/kg), and induced rat hepatic microsomes were isolated as described previously [31]. The substituted flavones (1 μM) were incubated with rat liver microsomes (25 μg protein/incubation) for 10 min as described previously [26], and EROD activity was determined spectrofluorimetrically [32].

Cell Growth, Induction, and Inhibition of EROD Activity

MCF-7 cells were grown in medium containing DME/F12 with 2.2 mg/mL sodium bicarbonate, 5% fetal bovine serum, and 10 nM antibiotic-antimycotic solution (Sigma Chemical Co., St. Louis, MO). For determination of EROD activities, the cells were first seeded into 60-mm petri dishes; 1 nM TCDD, different concentrations of the substituted flavones, and TCDD plus the flavones were dissolved in DMSO (0.1%) and added to the culture dishes when the cells reached 70% confluence. Cells were harvested 24 hr after chemical treatment, and EROD activity was determined fluorimetrically [32].

Northern Analysis for CYP1A1 mRNA Levels

The murine CYP1A1 cDNA probe was obtained from ATCC. The plasmid pGMB1.1 containing the mouse β-tubulin cDNA was a gift from Dr. Don Cleveland (Johns Hopkins University). Digestion of the plasmid yielded a 1.3 kb fragment that was used to detect β-tubulin mRNA. RNA from the cells treated with TCDD or substituted flavones for 24 hr was isolated, electrophoresed, transferred to a nylon membrane, and probed by autoradiography or using a Betagen Betascope 603 imaging system as described previously [33, 34].

Statistical Analysis

The statistical differences between different treatment groups were determined by ANOVA and Student's *t*-test, and the levels of probability are noted ($P < 0.05$ or $P < 0.01$). The data are expressed as means ± SEM or deviations SD for at least 3 determinations for each experimental point.

RESULTS

A series of ten 4′- and 3′,4′-substituted flavones were synthesized previously [27] and used in the present study. The results in Table 1 summarize the IC_{50} and K_i values obtained for the concentration-dependent displacement of [^{3}H]-TCDD from the rat cytosolic Ah receptor. The IC_{50} values for 4′-chloro-, 4′-bromo-, 4′-iodo-, and 3′,4′-dichloroflavone varied from 0.79 to 2.28 nM and were comparable to the IC_{50} value obtained for unlabeled TCDD (1.78 nM). The most active of the four compounds was 4′-iodoflavone (IC_{50} = 0.79 nM); all the remaining substituted flavones exhibited >100 times lower competitive binding affinities for the Ah receptor (IC_{50} values from 99 to 4697 nM). The K_i values exhibited comparable structure–activity relationships.

The effects of structure on ligand-induced transformation of the rat hepatic cytosolic Ah receptor were determined using gel mobility shift assays followed by quantitating the transformed Ah receptor-[^{32}P]DRE complex using a Betagen Betascope 603 blot analyzer. The effects of a 50 μM concentration of the substituted flavones were compared with the intensity of the retarded band observed with 5 nM TCDD. The intensity of the retarded band observed for 50

TABLE 1. Competitive binding of substituted flavones to rat hepatic cytosol Ah receptor and their activity to induce transformation of the rat cytosolic Ah receptor

Compound				DRE binding†
R (3′)	R (4′)	IC_{50}* (nM)	K_i* (nM)	(%)
H	H	216 ± 110	138	31 ± 6.0
H	OCH_3	99 ± 45	63	28 ± 7.7
H	NH_2	362 ± 128	232	ND‡
H	Cl	1.40 ± 0.4	0.92	351 ± 67
H	Br	1.50 ± 0.7	0.96	99 ± 10
H	NO_2	4697 ± 1991	2453	ND
NO_2	Cl	1935 ± 1139	1240	2.4 ± 1.2
NH_2	OH	738 ± 371	506	ND
Cl	Cl	2.28 ± 0.5	1.47	75 ± 12
H	I	0.79 ± 0.27	0.50 ± 0.35	240 ± 24

* The IC_{50} values (means ± SEM for 4 separate determinations) were determined in competitive binding studies using 1 nM [^{3}H]TCDD as the radioligand and different concentrations of unlabeled competitor as described in Materials and Methods; K_i values were calculated from the IC_{50} values.

† DRE binding was also determined using rat hepatic cytosol as described [26]; the results (means ± SEM for 4 separate determinations) are expressed as DRE binding observed for 50 μM concentrations of the flavones as a percentage of that observed using 5 nM TCDD in the transformation assay.

‡ ND = non-detectable.

μM 4′-chloroflavone was 3.5-fold higher than that observed for 5 nM TCDD; 50 μM 4′-bromo, 3′,4′-dichloro-, and 4′-iodoflavone all induced formation of a retarded band that was 75–240% of the intensity observed for 5 nM TCDD. The intensity of the retarded band induced by 50 μM concentrations for the remaining substituted flavones was <32% of that observed for 5 nM TCDD.

The results in Table 2 summarize the effects of 0.05, 0.5, 5.0 and 50 μM concentrations of the flavones on 5 nM TCDD-induced transformation of the rat cytosolic Ah receptor complex as determined by gel electrophoretic mobility shift assays and quantitation of the specifically bound retarded band (see Fig. 1). This study also included results for the methoxy-nitro/aminoflavones since these data were not reported previously [26]. All of the flavones significantly inhibited TCDD-induced transformation at some concentration. For example, 4′-methoxy- and 4′-iodoflavone inhibited 85 and 100% of TCDD-induced transformation at the lowest concentration (0.05 μM) used in this study. Several other substituted flavones inhibited >90% of TCDD-induced transformation at one or more concentrations, and these include flavone (5.0 μM), 4′-methoxyflavone (0.5 and 5.0 μM), 4′-aminoflavone (5.0 and 50 μM), 4′-nitroflavone (50 μM), 4′-chloro-3′-nitroflavone (5.0

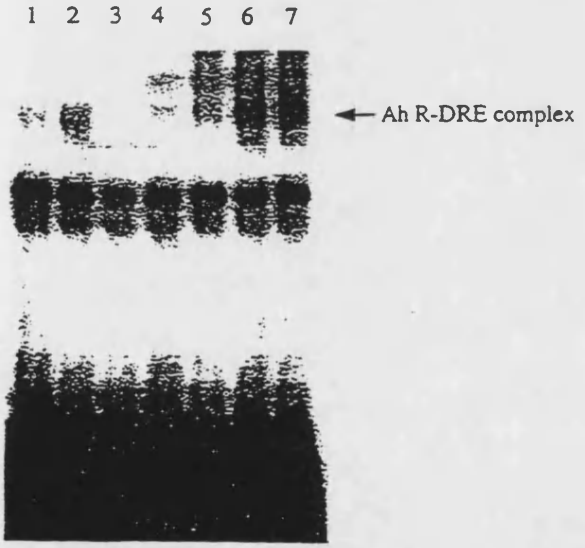

FIG. 1. Effects of TCDD, 4′-iodoflavone, and their combination on transformation of the rat heptic cytosolic Ah receptor. Rat hepatic cytosol was treated with DMSO (lane 1), 5 nM TCDD (lane 2), 5 nM TCDD plus 0.05 (lane 3), 0.5 (lane 4), 5 (lane 5), or 50 μM (lane 6) 4′-iodoflavone, and 50 μM 4′-iodoflavone alone (lane 7), and then incubated with [^{32}P]DRE; levels of the transformed DRE-Ah receptor complex were determined by gel electromobility shift assays as described in Materials and Methods. The results for the substituted flavones are summarized in Tables 1 and 2. The autoradiogram illustrated in this figure is the result of a single experiment using 4′-iodoflavone. The intensity of the specifically bound Ah receptor-DRE complex (see arrow) was non-detectable after incubation with a 200-fold excess of unlabeled DRE.

μM), 3′-amino-4′-hydroxyflavone (50.0 μM), 4′-iodoflavone (0.05 and 0.5 μM, Fig. 1), 4′-methoxy-3′-nitroflavone (50 μM), 3′-methoxy-4′-nitroflavone (5.0 and 50 μM), and 4′-amino-3′-methoxyflavone (5.0 and 50 μM). There were no apparent correlations between structure-Ah receptor binding (Table 1) and structure-inhibitory (TCDD-induced transformation) activities among the substituted flavones.

The Ah receptor agonist and antagonist activities of the substituted flavones were determined in Ah-responsive MCF-7 human breast cancer cells. At flavone concentrations from 0.01 to 10 μM, induction of EROD activity varied from 0 to 44% of the response observed for 1 nM TCDD (Fig. 2). In MCF-7 cells cotreated with 1 nM TCDD plus 0.01, 0.1, 1.0 or 10 μM concentrations of the substituted flavones, there was a concentration-dependent decrease in TCDD-induced EROD activity as illustrated in Fig. 2. All of the compounds caused a >90% decrease in induced EROD activity at concentrations varying from 0.1 to 10 μM, and the IC_{50} values for this inhibitory response are summarized in Table 3.

Previous studies have demonstrated that flavones and substituted flavones inactivate P4501A1-dependent activities by competitively interacting with the substrate binding site [26, 35–37], and therefore the effects of the substituted flavones on CYP1A1-dependent EROD activity were determined. Hepatic microsomes from Long-Evans rats treated with 5 μg/kg TCDD were incubated for 10 min with a 1 μM concentration of the substituted flavones in the presence or absence of NADH/NADPH. EROD activity was then determined, and the results are summarized in Fig. 3. All of the compounds inhibited EROD activity in the presence or absence of the reduced nucleotide cofactors, and the pattern of inhibition for the flavones was comparable, although there were significant structure-dependent differences in potency of their inhibitory effects. These data were similar to those reported previously for the 3′- and 4′-methoxy-amino/nitroflavones [26].

The Ah receptor agonist and antagonist activities of the substituted flavones were also investigated in MCF-7 cells by determining the induction of CYP1A1 mRNA levels by 1 nM TCDD, 1 μM substituted flavone, and 1 nM TCDD plus 1 μM substituted flavone. Relative CYP1A1 mRNA levels were compared with levels observed after treatment with 1 nM TCDD alone. The results (Table 3) demonstrate that 1 μM concentrations of 4′-amino-, 4′-bromo-, 4′-chloro-, 4′-iodo-, and 3′,4′-dichloroflavone induced CYP1A1 mRNA levels between 28 and 112% of that observed for 1 nM TCDD. CYP1A1 mRNA levels induced by the remaining compounds were <8% of that observed for 1.0 nM TCDD. In cells cotreated with 1 nM TCDD plus a 1 μM concentration of the substituted flavones, there was no significant decrease in CYP1A1 mRNA levels compared with cells treated with 1 nM TCDD alone. With the exception of the haloflavones, the remaining compounds were relatively weak inducers of CYP1A1 mRNA, and results of cotreatment studies suggest that these congeners

TABLE 2. Effects of substituted flavones on TCDD-induced transformation of the rat hepatic cytosolic Ah receptor

Compound		% of TCDD-induced transformation*			
		Flavones (μM)			
R (3')	R (4')	0.05	0.5	5.0	50
H	H	90 ± 14	24 ± 6.4†	ND†‡	27 ± 5.3†
H	OCH_3	15 ± 11†	ND†‡	ND†‡	37 ± 13†
H	NH_2	96 ± 25	48 ± 9.4†	ND†‡	ND†‡
H	Cl	58 ± 21†	39 ± 15†	137 ± 27	338 ± 92
H	Br	43 ± 6.4†	67 ± 6.4†	141 ± 17	91 ± 18
H	NO_2	83 ± 13	97 ± 18	37 ± 13†	ND†‡
NO_2	Cl	99 ± 15	106 ± 21	19 ± 8†	10 ± 6†
NH_2	OH	99 ± 16	87 ± 4	43 ± 9†	ND†‡
Cl	Cl	57 ± 8†	31 ± 7.4†	69 ± 5.3†	81 ± 12
H	I	ND†‡	ND†‡	105 ± 25	240 ± 108
NO_2	OCH_3	112 ± 10	86 ± 15	15 ± 5†	ND†‡
NH_2	OCH_3	79 ± 8.6†	29 ± 9.4†	14.4 ± 9.9†	73 ± 19
OCH_3	NO_2	58 ± 8.0†	ND†‡	ND†‡	ND†‡
OCH_3	NH_2	99 ± 14	50 ± 15†	ND†‡	ND†‡

* The effects of 5 nM TCDD plus 0.05, 0.5, 5.0 or 50 μM concentrations of the substituted flavones on transformation of rat hepatic cytosolic Ah receptor were determined by gel electrophoretic mobility shift assays as described in Materials and Methods. The intensities of the specifically bound retarded bands were normalized to that observed for 5 nM TCDD alone (100%). Results are expressed as means ± SEM for at least 4 determinations.

† Significantly lower ($P < 0.05$) than observed for 5 nM TCDD alone.

‡ ND = non-detectable.

were not Ah receptor antagonists. Of the remaining haloflavones, 4'-iodoflavone was the most effective inhibitor of TCDD-induced transformation at low concentrations (Fig. 1 and Table 2), and therefore the effects of 4'-iodoflavone (0.01 to 1.0 μM) on induction of CYP1A1 mRNA were investigated further. The results (Fig. 4) show that 0.01 and 0.1 μM 4'-iodoflavone significantly inhibited induction of CYP1A1 mRNA by 1 nM TCDD in MCF-7 cells.

DISCUSSION

Several different structural classes of compounds bind to the cytosolic Ah receptor, and these include TCDD and related polyhalogenated aromatics, polynuclear aromatic hydrocarbons (PAHs), indole-3-carbinol and related hetero-PAHs, phenanthrolines, phenanthridinones, benzocoumarins and various substituted flavonoids [23, 38–45]. Systematic structure–activity relationships have been reported for the halogenated aromatics [2, 20, 21], and there was a rank order correlation between their cytosolic Ah receptor binding affinities and their potencies as Ah receptor agonists for *CYP1A1* induction and other Ah receptor-mediated biochemical and toxic responses. Moreover, similar structure-dependent responses have been reported for other steps associated with the Ah receptor signalling pathway, and these include ligand-induced transformation of the cytosolic Ah receptor and formation of the nuclear Ah receptor complex [15, 22]. There are only limited studies on structure-dependent responses associated with ligand-induced activation of the Ah receptor for other structural classes of compounds that bind to the Ah receptor. Lu and coworkers [26] recently reported that 3',4'-substituted methoxy-amino/nitro flavones exhibited structure-dependent Ah receptor agonist and antagonist activity. Both 3'-methoxy-4'-nitro- and 3'-methoxy-4'-aminoflavone were Ah receptor antagonists, whereas the 4'-methoxy analogs were more active as Ah receptor agonists. This study reports a more extensive structure–activity study on several substituted flavones utilizing various assays that are associated with the Ah-responsiveness of halogenated aromatics.

The results in Table 1 show that the competitive binding affinities for the rat cytosolic Ah receptor vary from 0.79 nM for 4'-iodoflavone to 4697 nM for 4'-nitroflavone. Previous Ah receptor binding studies with various substituted polyhalogenated aromatics, such as 4'-substituted-2,3,4,5-tetrachlorobiphenyls, 2-substituted-3,7,8-trichlorodibenzo-*p*-dioxins, and 7-substituted-2,3-dichlorodibenzo-*p*-dioxins, showed that the halogen-substituted analogs (I, Br and Cl) exhibit the lowest K_D values [46–49], and, in this study, the 4'-iodo-, 4'-chloro-, 4'-bromo-, and 3',4'-dichloroflavone also exhibited high binding affinities and K_D values that were comparable to the value reported for TCDD (K_D = 1.28 nM) (Table 1). Despite the correlations observed for the halogenated aromatics and halogenated flavones, Lu and coworkers [26] have shown previously that 3'-methoxy-4'-nitroflavone also binds with high affinity to the rat cytosolic Ah receptor (K_D = 2.27 nM); in contrast, methoxy- and nitro-substituents do not enhance binding of substituted aromatics to the Ah receptor [50]. Moreover, forsubstituted halogenated aromatics, electron-donating substituents are invariably more deactivating for Ah receptor binding than electron-withdrawing substituents

[46–49]. For example, the receptor binding IC_{50} values for 7-amino- and 7-hydroxy-2,3-dichlorodibenzo-*p*-dioxin were 2.88×10^{-5} and 4.47×10^{-6} M, respectively, whereas the IC_{50} values for the electron-withdrawing nitro-substituted analog was 4.6×10^{-7} M. In contrast, 4′-nitroflavone exhibited lower binding affinities (IC_{50} = 4697 nM) than 4′-aminoflavone or 3′-amino-4′-hydroxyflavone (362 and 738 nM, respectively). The reasons for substituent-dependent differences in Ah receptor binding affinities of the substituted flavones and halogenated aromatics are unknown.

The relative potencies of substituted flavones (50 μM) to induce transformation of rat hepatic cytosolic receptor were compared with that observed for 5 nM TCDD using a gel mobility shift assay. The results (Table 1) showed the 4′-chloro-, 4′-iodo-, 4′-bromo, and 3′,4′-dichloroflavones that

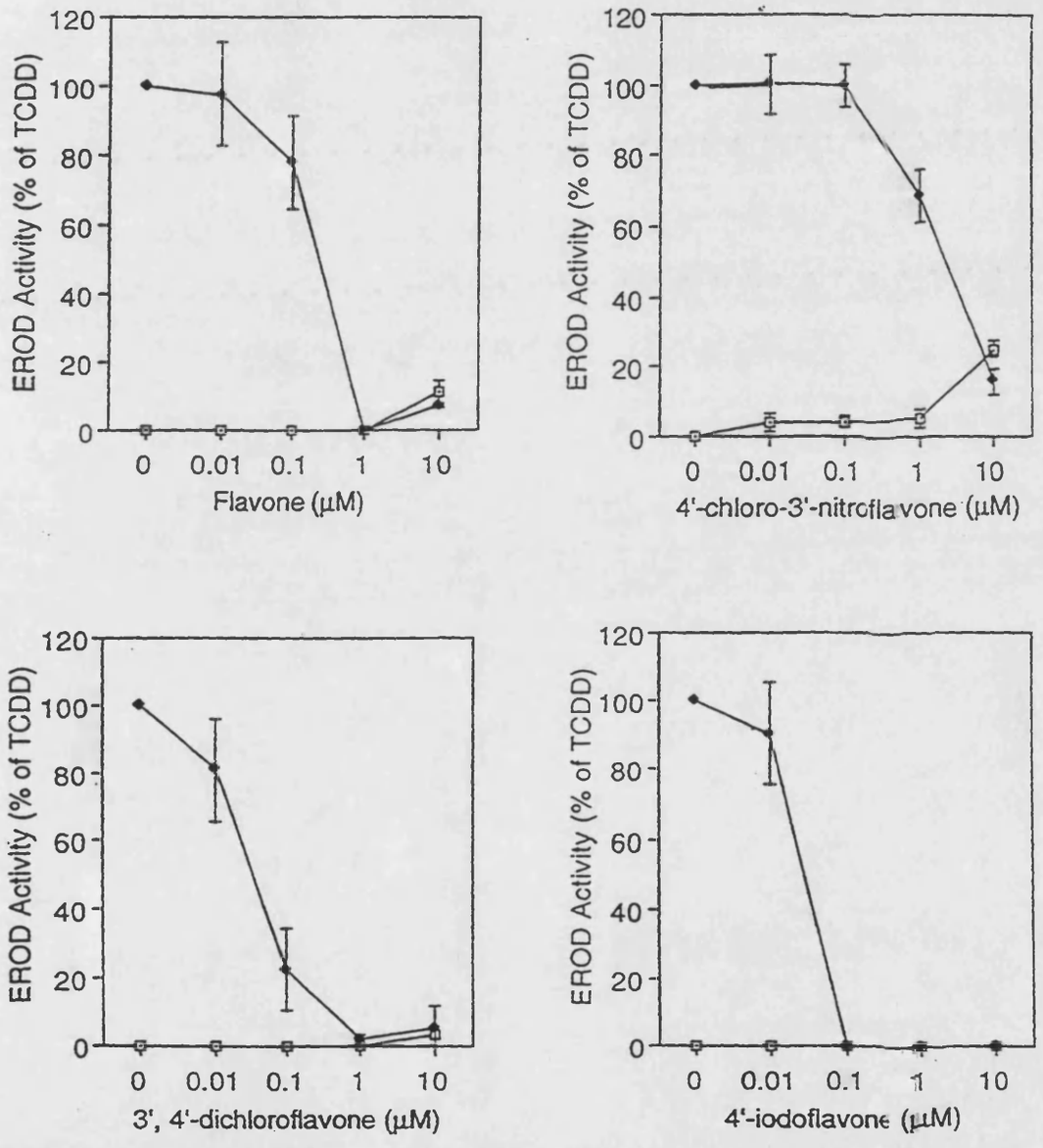

FIG. 2. Induction of EROD activity by TCDD, substituted flavones, and their combinations. MCF-7 cells were incubated for 24 hr with different concentrations of substituted flavones (□) alone or 1 NM TCDD plus different concentrations of the substituted flavones (♦), and EROD activity was determined as described in Materials and Methods. Representative data obtained for flavone (upper left), 4′-chloro-3′-nitroflavone (upper right), 3′,4′-dichloroflavone (lower left) and 4′-iodoflavone (lower right) are present. Results are expressed as means ± SD for 3 separate determinations for each data point. EROD activities in cells treated with 10 μM concentrations of the substituted flavones as a percent of the value observed for 1 nM TCDD (100%) were: 11 ± 3, 17 ± 1.6, 21 ± 2.7, 44 ± 9, 25 ± 0.3, non-detectable, 25 ± 2.4, 3.5 ± 2.9, 3.1 ± 2.7%, and non-detectable for flavone, 4′-methoxy-, 4′-amino-, 4′-chloro-, 4′-bromo-, 4′-nitro-, 4′-chloro-3′-nitro-, 3′-amino-4′-hydroxy-, 3′,4′-dichloro-, and 4′-iodoflavone, respectively.

TABLE 3. Substituted flavones: Induction of CYP1A1 mRNA levels and inhibition of TCDD-induced CYP1A1 mRNA levels and EROD activity in MCF-7 cells*

Compound		Relative % CYP1A1 mRNA		Inhibition of induced EROD activity (IC_{50}, M)
R (3')	R (4')	Flavone (1 μM)	Flavone (1 μM) + TCDD (1 nM)	
H	H	3.5 ± 2.9	96 ± 5.4	2.9×10^{-7}
H	OCH_3	NDt	100 ± 29	4.0×10^{-7}
H	NH_2	28 ± 9.8	98 ± 35	5.7×10^{-7}
H	Cl	70 ± 6.4	100 ± 12	1.3×10^{-7}
H	Br	32 ± 3.2	99 ± 10	9.9×10^{-8}
H	NO_2	2.8 ± 0.6	95 ± 21	2.2×10^{-6}
NO_2	Cl	4.4 ± 4.4	86 ± 29	1.8×10^{-6}
NH_2	OH	7.1 ± 4.3	83 ± 4.7	6.1×10^{-6}
Cl	Cl	75 ± 21	85 ± 47	4.7×10^{-8}
H	I	112 ± 67	141 ± 5.6	2.4×10^{-8}

* mRNA was extracted from MCF-7 cells 24 hr after treatment with 1 nM TCDD or 1 nM TCDD + the flavones (1 μM). mRNA was analyzed by northern blot analysis as described in Materials and Methods. CYP1A1 mRNA levels were normalized to β-tubulin mRNA, and relative percent mRNA levels were compared with that observed for 1 nM TCDD (100%). The results are expressed as means ± SD for at least 3 determinations for each data point. The IC_{50} values for this inhibitory response were determined by log-logit plots of the percent of maximal induced responses observed in cells cotreated with 1 nM TCDD plus different concentrations of the flavones. Maximal (100%) EROD activity was obtained with 1 nM TCDD alone (110 pmol/min/mg).

† ND = not detectable.

exhibited high competitive binding for the Ah receptor binding (IC_{50} = 0.79 to 2.28 nM) induced the highest levels of transformed cytosolic Ah receptor complex, which were 75–351% of that observed for 5 nM TCDD. All of these compounds exhibited receptor binding IC_{50} values comparable to that of TCDD, and with the exception of 4'-chloroflavone, their activity to induce transformation of the cytosolic Ah receptor was > 5000-fold lower than TCDD. The remaining flavones exhibited lower binding affinities for the Ah receptor (Table 1) and induced relatively low levels of transformed receptor (Table 2). However, results of previous studies [26] showed that 2 substituted flavones, namely 3'-methoxy-4'-nitro- and 4'-amino-3'-methoxyflavone, which bound competitively with high affinity to the Ah receptor (IC_{50} = 2.27 and 86.1 nM, respectively), induced minimal transformation (<6%) of the cytosolic Ah receptor. Thus, there was no correlation between receptor binding affinities and activity to induce transformation for these substituted flavones.

The structure-dependent Ah receptor agonist activities of the substituted flavones were investigated by determining induction of CYP1A1 mRNA levels and EROD activity in MCF-7 cells (Table 3). A 10 μM concentration of the substituted flavones induced 0–44% of EROD activity observed for 1 nM TCDD (Fig. 2), and there was no correlation between structure-binding and structure-induction activities. One micromolar concentrations of 4'-amino-, 4'-chloro-, 4'-bromo-, 3',4'-dichloro-, and 4'-iodoflavone induced mRNA levels that were 28–112% of levels observed for 1 nM TCDD. Moreover, results of previous studies showed that the 3'-amino-4'-methoxyflavone also induces CYP1A1 mRNA levels [26]. With the exception of 4'-aminoflavone, the most active inducers of CYP1A1 mRNA levels were those congeners that bound with high affinity to the Ah receptor and induced transformation of the cytosolic Ah receptor complex. In contrast, 4'-aminoflavone exhibited relatively low Ah receptor binding affinity and did not induce receptor transformation. 3'-Amino-4'-methoxyflavone bound with higher affinity to the Ah receptor (IC_{50} = 19 nM) but also did not induce receptor transformation (Table 2), showing that for these flavones there was no correlation between their activities to induce transformation and *CYP1A1* gene expression. This is in contrast to previous studies wtih TCDD and related halogenated aromatics.

Lu and coworkers [26] previously reported that 3'-methoxy-4'-nitroflavone exhibited unusual properties since this compound exhibited high binding affinity for the Ah receptor (IC_{50} = 2.27 nM) but did not induce receptor transformation or CYP1A1 mRNA levels in MCF-7 cells. However, in MCF-7 cells cotreated with 1 nM TCDD plus 1 μM 3'-methoxy-4'-nitroflavone, there was a significant inhibition of EROD activity and CYP1A1 mRNA levels induced by TCDD. Therefore, the second phase of this study investigated the potential Ah receptor antagonist activity of the substituted flavones by determining their effects on TCDD-induced transformation of the cytosolic Ah receptor and on induction of EROD activity and CYP1A1 mRNA levels in MCF-7 cells.

Previous studies with two different structural classes of Ah receptor antagonists typified by MCDF and αNF showed that inhibition of CYP1A1 mRNA levels or EROD activity is also accompanied by inhibition of TCDD-induced transformation of the cytosolic Ah receptor [22, 33,

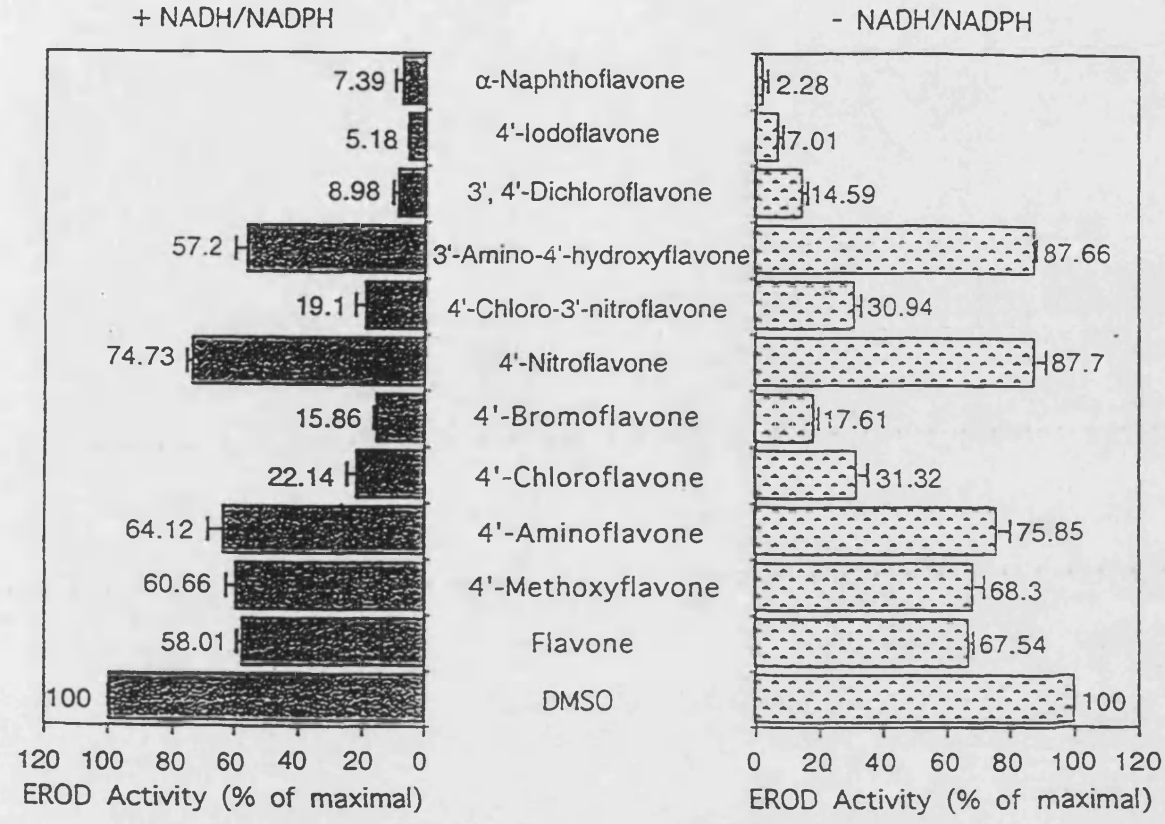

FIG. 3. *In vitro* **inhibition of EROD activity by substituted flavones. TCDD-induced rat hepatic microsomes were incubated with a 1 μM concentration of the substituted flavones for 10 min, and EROD activity was determined fluorimetrically as described in Materials and Methods. EROD activity after incubation with the flavones is expressed as a percentage of activity observed after incubation of microsomes with DMSO (solvent control). Control activity = 5824 pmol/min/mg protein. The incubations were carried out in the presence (left) or absence (right) of NADH/NADPH cofactors. The results are expressed as means ± SD for 3 separate determinations.**

34, 51, 52]. The results in Table 2 show that all of the substituted flavones significantly inhibited TCDD-induced transformation and with the exception of 4'-chloroflavone, 4'-bromoflavone, 3',4'-dichloroflavone, and 3'-amino-4'-methoxyflavone, >90% of induced transformation was inhibited at some concentration (Table 2 and Fig. 1). Initial studies also showed that in cells cotreated with 0.01 to 10 μM concentrations of the substituted flavones plus 1 nM TCDD, there was a concentration-dependent decrease in induced EROD activity (Table 3 and Fig. 2). The IC_{50} values for inhibition of EROD activity in MCF-7 cells varied from 0.024 μM for 4'-iodoflavone to 6.14 μM for 3'-amino-4'-hydroxyflavone. Previous studies showed that the isomeric methoxy-nitro/aminoflavones also inhibited induced EROD activity, and this was due, in part, to *in vitro* inhibition of CYP1A1 [26]. The results summarized in Fig. 3 show that after incubation of the substituted flavones (1 μM) with hepatic microsomes in the presence or absence of reduced nucleotide cofactors, there was significant inhibition of EROD activity by all the substituted flavones. These results are consistent with previous reports which show that various flavones bind to cytochrome P450 and/or inhibit P450-mediated activities [26, 35–37]. Thus, inhibition of induced EROD activity in MCF-7 cells by the substituted flavones (Fig. 3) may be due, in part, to competition with ethoxyresorufin for substrate binding sites on the enzyme.

Since previous studies showed that 4'-amino-3'-methoxy- and 3'-methoxy-4'-nitroflavone antagonize induction of both EROD activity and CYP1A1 mRNA levels induced by TCDD in MCF-7 cells [26], inhibition of induced CYP1A1 mRNA levels by the substituted flavones (1 μM) was also investigated. Only minimal inhibitory effects were observed, even though most of the compounds (with the exception of the haloflavones) exhibited relatively weak induction of CYP1A1 mRNA at the 1 μM concentration. Of the remaining haloflavones, 4'-iodoflavone was the most effective inhibitor of TCDD-induced transformation of the Ah receptor (Table 2), and interactions of 4'-iodoflavone plus TCDD showed that 0.01 and 0.1 μM concentrations of the former compound inhibited induction of CYP1A1 mRNA by 1 nM TCDD (Fig. 4). Thus, 4'-iodoflavone exhibited both Ah receptor agonist and antagonist activities.

Previous studies with halogenated aromatics and various substituted analogs showed that their activity as Ah receptor agonists was structure dependent, and that Ah receptor

FIG. 4. Northern blot analysis of CYP1A1 mRNA levels in MCF-7 cells treated with TCDD, 4′-iodoflavone, and their combinations. mRNA was extracted from MCF-7 cells 24 hr after treatment with DMSO (lane 1), 1 nM TCDD (lane 2), 0.01, 0.1 and 1.0 μM 4′-iodoflavone (lanes 3 through 5, respectively), and 1 nM TCDD plus 0.01, 0.1 or 1.0 μM 4′-iodoflavone (lanes 6 through 8, respectively). mRNA levels were determined by northern blot analysis and expressed relative to β-tubulin mRNA as described in Materials and Methods. The mRNA levels in lanes 1 through 8 are expressed relative to those observed after treatment with 1 nM TCDD (100%): non-detectable, 100, 2.2 ± 2.6, 46 ± 20, 112 ± 67, 42 ± 4.9, 33 ± 11, and 141 ± 5.6 (lanes 1 through 8, respectively; means ± SD for at least 3 determinations); 0.01 and 0.1 μM 4′-iodoflavone significantly ($P < 0.05$) inhibited induction of CYP1A1 mRNA levels by TCDD.

binding affinities and ligand-induced transformation activities correlated with CYP1A1 induction potencies [2, 20]. Despite the unusually high affinity of several substituted flavones for the cytosolic Ah receptor, structure–activity correlations for these compounds were inconsistent. These data suggest that various structural classes of Ah receptor ligands induce different conformational changes in the cytosolic Ah receptor complex which differentially influence individual steps in the Ah receptor-mediated signal transduction pathway. Moreover, the results for the substituted flavones suggest that within this structural class of compounds, various substituent groups can affect markedly the activity of each individual congener as an Ah receptor agonist or antagonist. These substituent-dependent differences in the activity of the flavones may be related to ligand-induced conformational changes in the Ah receptor complex and also provide support for the proposed existence of more than one form of the Ah receptor [26, 53–55]. The existence of multiple conformations or forms of the Ah receptor complex is speculative, and future studies will utilize radiolabeled synthetic flavones that can be used to investigate both the direct binding and photoaffinity labeling of the Ah receptor.

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PUBLICATION 72

Labelled Compounds of Interest as Antitumour Agents - V.
Syntheses of [^{18}O]-5-Methylisoquinolinone and
1-(Furan-2-yl-[^{18}O]-methoxy)-5-methylisoquinoline

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LABELLED COMPOUNDS OF INTEREST AS ANTITUMOUR AGENTS - V[1]. SYNTHESES OF [^{18}O]-5-METHYLISOQUINOLINONE AND 1-(FURAN-2-YL-[^{18}O]-METHOXY)-5-METHYLISOQUINOLINE

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SUMMARY

Treatment of 2-methylcinnamic acid with $H_2{}^{18}O$ at 100°C under acidic conditions leads to high incorporation of ^{18}O by exchange. Methods have been developed for chemically and isotopically efficient conversion to the corresponding [carbonyl-^{18}O] methyl ester, to [^{18}O]-5-methylisoquinolinone (an inhibitor of DNA repair) and to 1-(furan-2-yl-[^{18}O]-methoxy)-5-methylisoquinoline.

Introduction

The enzyme poly(ADP-ribose)polymerase (PARP, EC 2.4.2.30) catalyses the transfer of ADP-ribose units from NAD^+ to form a polyanionic polymer on histones and other acceptor proteins near the site of damage to DNA[2-4]. Inhibition of PARP leads to inhibition of the processes of repair of damaged sites in DNA[5-7] and thus to potentiation of the antitumour effects of many forms of radiotherapy[8-11] and chemotherapy[12-14]. Potent inhibitors of PARP include benzamides and analogues in which the conformation of the amide is constrained relative to the aromatic ring, by incorporation into a lactam[8,15,16] or by hydrogen-bonding[16]. Of these lactams, 5-substituted isoquinolinones have been shown[8,15] to be among the inhibitors with the greatest potency. During our studies on drugs which sensitise tumours to the cytotoxic effects of radiotherapy and chemotherapy[17], we required a 5-substituted isoquinolinone PARP inhibitor labelled with ^{18}O at the carbonyl oxygen. In this paper, we report our development of syntheses of methyl [carbonyl-^{18}O]-*E*-3-(2-methylphenyl)propenoate **4**, a model intermediate to check satisfactory isotopic incorporation, of [^{18}O]-5-methylisoquinolinone **7** and of a potential pro-drug derivative, 1-(furan-2-yl-[^{18}O]-methoxy)-5-methylisoquinoline **10**.

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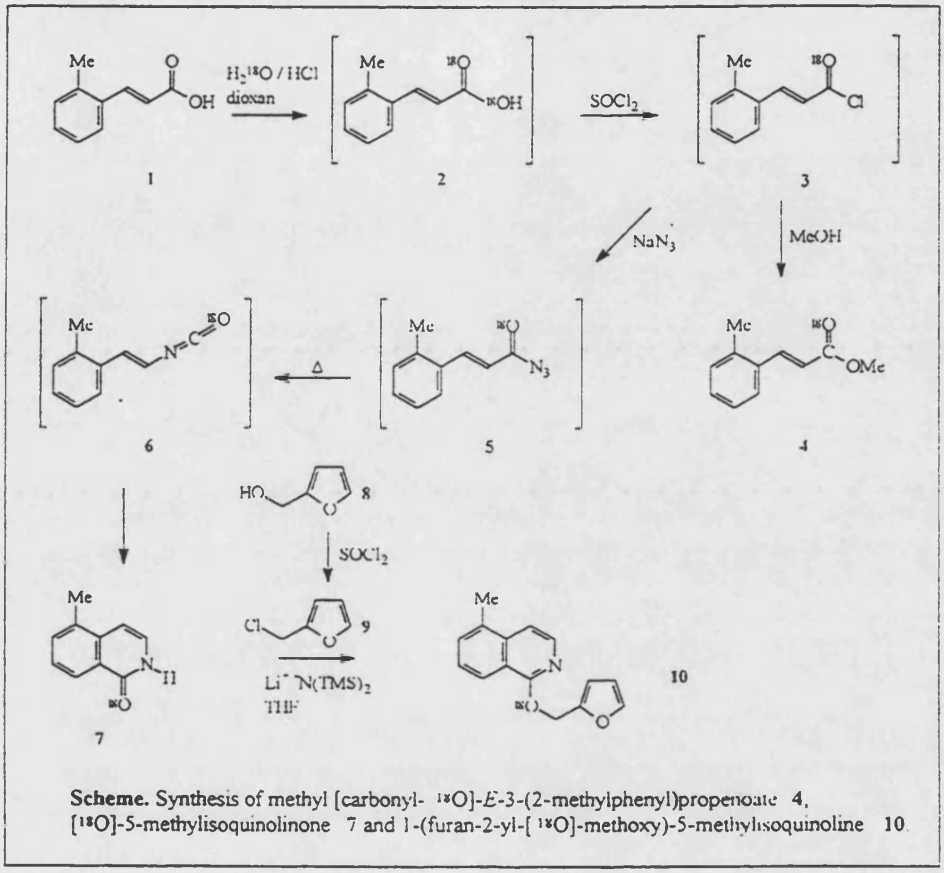
Results & Discussion

A convenient synthesis of isoquinolinones by Curtius rearrangement of cinnamyl azides and thermal cyclisation, in a one-pot process, has been described by Eloy and Deryckere[15]. The Curtius reaction involves a true rearrangement of the intermediate acyl nitrene to form the isocyanate. Thus ^{18}O present in the carbonyl group should be retained in the isocyanate. The subsequent mechanistic steps, thermal isomerisation of the C=C and cyclisation, also take place under conditions where the oxygen atom should be retained in the isoquinolinone. The synthetic route, therefore, requires the ^{18}O-labelled 2-methylcinnamyl azide 5 as a starting material for the synthesis of the ^{18}O-labelled 5-methylisoquinolinone 7.

We considered that 5, in turn could be derived from the corresponding ^{18}O-labelled acid chloride 3 and the ^{18}O-labelled carboxylic acid 2. An initial series of model experiments was devised to develop a procedure for incorporation of ^{18}O into 2-methylcinnamic acid 1, forming 2, and for conversion to the acid chloride 3, under conditions which would not permit loss of ^{18}O by exchange. ^{18}O was incorporated by heating the carboxylic acid 1 in 1,4-dioxan with twenty equivalents of $H_2{}^{18}O$ under acidic conditions to promote exchange. The solvent and excess water were removed by distillation in a closed system to give the isotopomer 2. Since this acid is capable of losing ^{18}O by exchange with atmospheric water, it was rapidly converted to the corresponding acid chloride 3 with thionyl chloride. To check the level of incorporation of ^{18}O, the acid chloride was quenched with methanol. Mass spectrometry showed the ester 4 to contain 8.0 ± 0.7% ^{18}O in the carbonyl group, confirming that the exchange process had proceeded satisfactorily and that little ^{18}O had been lost during the subsequent reactions.

Now that the initial exchange reaction had been developed and conversion to the acid chloride 3 had been established, attention was focussed on the main isoquinolinone-forming process. The ^{18}O-labelled acid chloride 3 was prepared as before but on a multimillimole scale. The acid chloride 3 should not be subject to exchange of ^{18}O under aqueous conditions without hydrolysis, so conversion to the acyl azide 4 was effected by treatment with sodium azide in aqueous acetone. Any material which may have been hydrolysed to the acid would not be converted to the acyl azide by this process. The Curtius reaction was carried out in boiling diphenyl ether at *ca.* 260°C, forming the isocyanate 6, which was isomerised *E*→*Z* and cyclised under the same conditions, giving the ^{18}O-labelled 5-methylisoquinolinone 7 in good yield. Again, the isotopic composition was determined by mass spectrometry; 7 was shown to contain 8.0% ^{18}O at the carbonyl oxygen. This represents the same incorporation as was seen for the methyl 2-methylcinnamate 4, indicating no loss of ^{18}O in steps after the acid chloride.



Scheme. Synthesis of methyl [carbonyl- ^{18}O]-*E*-3-(2-methylphenyl)propenoate **4**, [^{18}O]-5-methylisoquinolinone **7** and 1-(furan-2-yl-[^{18}O]-methoxy)-5-methylisoquinoline **10**.

For the synthesis of the *O*-furanylmethyl derivative **10**, two synthetic approaches were possible; (*i*) reaction of furan-2-[^{18}O]-methanol, as its alkoxide, with 1-chloro-5-methylisoquinoline or (*ii*) alkylation of the anion of the isoquinolinone **7** with an electrophilic furanylmethyl compound. The former was discounted, as this would require development of a synthesis of a new ^{18}O-labelled intermediate, the furanmethanol. Nevertheless, the reaction of unlabelled sodium furanmethoxide with 1-chloroisoquinoline was investigated but was found to lead only to destruction of the furan at the high temperature of the reaction. The second approach also presented some problems, as furanylmethyl electrophiles are notoriously unstable.

2-Chloromethylfuran **9** was prepared by treatment of the corresponding alcohol **8** with thionyl chloride by the general method of Tarrago *et al*[19]. This unstable material was allowed to react with the lithium anion of the ^{18}O-labelled 5-methylisoquinolinone **7** at reflux in tetrahydrofuran. To counteract the loss of **8** by decomposition during this alkylation, it was used in large excess. The ^{18}O-labelled furanylmethoxyisoquinoline **10** was obtained in good chemical yield and with excellent

isotopic enrichment (8.0% ^{18}O). The equivalence of the isotopic enrichment of 7 and 10 demonstrates that no loss of ^{18}O has occurred during the alkylation.

Conclusion

Efficient techniques have been developed for the incorporation of ^{18}O into a *Ar*-substituted cinnamic acid by exchange with $H_2{}^{18}O$ and for subsequent conversion to the carbonyl-^{18}O-labelled isoquinolinone 7 and the furanylmethyl ether 10. These methods should be applicable to syntheses of other ^{18}O-labelled cinnamate esters and to other isoquinolinones which do not bear strong electron-withdrawing groups. The results of biological and biomimetic studies with 7 and 10 will be reported elsewhere.

Experimental

Jeol GX270 and EX400 instruments furnished the NMR spectra of solutions in $CDCl_3$; the internal standard was tetramethylsilane. Melting points are uncorrected. Solvents were evaporated under reduced pressure. The chromatographic stationary phase was silica gel. Brine refers to a saturated solution of sodium chloride in water. [^{18}O]-Water (10 atom %) was obtained from the Aldrich Chemical Company.

Methyl [carbonyl-^{18}O]-*E*-3-(2-methyl)phenylpropenoate (4). *E*-3-(2-methyl)phenylpropenoic acid 1 (53 mg, 330 μmol) was boiled under reflux with [^{18}O]-water (120 μl, 10 atom %) and hydrogen chloride (1.0 M in diethyl ether, 150 μl) in dry 1,4-dioxan (1.5 ml) for 24 h. The solvents and excess reagent were distilled off under nitrogen and the residue was stirred with thionyl chloride (3.0 ml) and dimethylformamide (10 μl) for 16 h. The excess reagent was evaporated. Methanol (2.0 ml) was added and the mixture was stirred for 1 h. Chromatography (ethyl acetate) yielded 4 (46 mg, 79%) as a pale yellow oil (lit.[20] unlabelled compound is an oil): δ_H 2.43 (3 H, s, Ar-Me), 3.81 (3 H, s, OMe), 6.36 (1 H, d, J = 15.9 Hz, HC=C), 7.18-7.28 (3 H, m, Ar 3,4,5-H_3), 7.54 (1 H, d, J = 7.0 Hz, Ar 6-H), 8.00 (1 H, d, J = 15.9 Hz, C=CH); δ_C 19.74, 51.62, 118.8, 126.29, 126.35, 129.97, 130.73, 133.32, 137.59, 142.49, 167.40; *m/z* (EI) 178 (2.7%) (M), 176 (33%) (M), 163 (2.0%) (M - Me), 161 (23%) (M - Me), 147 (9%) (M - MeO), 145 (90%) (M - MeO).

[^{18}O]-5-Methylisoquinolinone (7). *E*-3-(2-methyl)phenylpropenoic acid 1 (450 mg, 2.8 mmol) was boiled under reflux with [^{18}O]-water (1.00 ml, 10 atom %) and hydrogen chloride (1.0 M in diethyl ether, 1.5 ml) in dry 1,4-dioxan (15 ml) for 12 h. The solvents and excess reagent were distilled off under nitrogen and the residue was stirred with thionyl chloride (5 ml) and dimethylformamide

(10 μl) for 1.5 h. The excess reagent was evaporated. The acid chloride **3**, in acetone (5 ml), was added to sodium azide (630 mg, 9.7 mmol) in water (2 ml) and acetone (1 ml) at 0°C. The mixture was stirred for 30 min at this temperature. The acyl azide **5** was extracted with dichloromethane and was washed with brine and was dried ($MgSO_4$ / $CaCl_2$). Diphenyl ether (3 ml) was added and the dichloromethane was evaporated at ambient temperature. The residue was added to boiling diphenyl ether (10 ml) during 10 min and the solution was boiled under reflux for 2 h. Evaporation and chromatography (ethyl acetate / hexane 1:1) gave **7** (270 mg, 61%) as a pale yellow solid: mp 178-180°C (lit.[18] mp 184-185°C for the unlabelled compound); δ_H 2.55 (3 H, s, Me), 6.71 (1 H, d, J = 7.3 Hz, 4-H), 7.25 (d, J = 6.4 Hz, 3-H), 7.40 (1 H, dd, J = 7.9, 7.3 Hz, 7-H), 7.52 (1 H, d, J = 7.0 Hz, 6-H), 8.30 (1 H, d, J = 7.9 Hz, 8-H); δ_C 19.17, 103.47, 125.18, 126.10, 126.39, 127.47, 133.45, 137.18, 164.90 (one C_q was not observed); *m/z* (EI) 161.0726 ($C_{10}H_9N^{18}O$ requires 161.0727) (8.7%) (M), 159.0682 ($C_{10}H_9N^{16}O$ requires 159.0684) (100%) (M).

1-(Furan-2-yl-[^{18}O]-methoxy)-5-methylisoquinoline (10). To furan-2-methanol **8** (1.00 g, 10 mmol) in chloroform (10 ml) was added pyridine (1.5 ml). The solution was cooled to -10°C and thionyl chloride (2.0 ml) in chloroform (20 ml) was added. The mixture was stirred at this temperature under nitrogen for 3 h. Hydrochloric acid (10%, 10 ml, 0°C) was added. The organic phase was washed with hydrochloric acid (10%, 0°C) and with aqueous sodium hydroxide (3%, 0°C). The solution was dried ($MgSO_4$ / K_2CO_3) and the solvent was evaporated at ambient temperature to give **9** (1.07 g, 89%) as an unstable orange liquid. The [^{18}O]-isoquinolinone **7** (250 mg, 1.6 mmol), in tetrahydrofuran (25 ml), was treated with lithium hexamethyldisilazide (1.0 M in tetrahydrofuran, 2.0 ml) and the mixture was stirred at ambient temperature for 1 h. 2-Chloromethylfuran **9** (1.00 g, 8.6 mmol), in tetrahydrofuran (25 ml) was added dropwise during 1 h at 0°C, followed by sodium iodide (20 mg). The mixture was boiled under reflux for 18 h. The evaporation residue, in ethyl acetate, was washed with water and with brine and was dried ($MgSO_4$). Evaporation and chromatography (ethyl acetate / hexane 1:5) gave **10** (224 mg, 60%) as a pale yellow oil which crystallised on standing: mp 84-86°C; δ_H 2.51 (3 H, s, Me), 5.19 (2 H, s, CH_2), 6.33 (1 H, dd, J = 3.1, 1.9 Hz, furan 4-H), 6.42 (1 H, d, J = 3.3 Hz, furan 3-H), 6.61 (1 H, d, J = 7.7 Hz, isoquinoline 4-H), 7.21 (1 H, d, J = 7.7 Hz, isoquinoline 3-H), 7.36 (2 H, m, isoquinoline 7-H + furan 5-H), 7.46 (1 H, d, J = 7.2 Hz, isoquinoline 6-H), 8.32 (1 H, d, J = 8.1 Hz, isoquinoline 8-H); δ_C 18.93, 44.33, 103.02, 109.42, 110.64, 110.83, 125.97, 126.55, 130.54, 133.08, 133.17, 135.90, 142.77, 149.78, 162.1; *m/z* (EI) 241.0990 ($C_{15}H_{13}N^{16}O^{18}O$ requires 241.0989) (3.6%) (M), 239.0943 ($C_{15}H_{13}N^{16}O_2$ requires 239.0946) (40%) (M), 81 (100%) (furan-CH_2).

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PUBLICATION 73

Labelled Compounds of Interest as Antitumour Agents - VI.
Isotopically Efficient Syntheses of [^{15}N]-Nitrothiophenecarboxamides

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LABELLED COMPOUNDS OF INTEREST AS ANTITUMOUR AGENTS - VI[1]. ISOTOPICALLY EFFICIENT SYNTHESES OF [^{15}N]-NITROTHIOPHENECARBOXAMIDES

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SUMMARY

Reaction of 3-cyanothiophene with one equivalent of potassium nitrate in concentrated sulphuric acid causes nitration, concurrent with hydrolysis of the nitrile, to give 5-nitrothiophene-3-carboxamide in high yield. Similarly, 2-cyanothiophene gives 4-nitrothiophene-2-carboxamide and 5-nitrothiophene-2-carboxamide, benzonitrile gives 3-nitrobenzamide and 4-methylbenzonitrile gives 4-methyl-3-nitrobenzamide. Extension of this process to use of potassium [^{15}N]-nitrate, formed from [^{15}N]-nitric acid (95% isotopic enrichment), enables preparation of the corresponding [^{15}N]-nitrothiophenecarboxamides in high isotopic yield.

Introduction

As part of a programme of synthesis and evaluation of nitro-heterocycles as radiosensitisers, bioreductively-activated cytotoxins and inhibitors of repair of DNA[2-6], we required the three possible isomeric nitro-thiophene-carboxamides with the functional groups in the 'meta' relationship. These compounds can be regarded as heterocyclic analogues of 3-nitrobenzamide. We also required the corresponding [^{15}N]-nitro compounds for metabolic and other studies. Thiophenes are usually nitrated using acetyl nitrate in acetic acid or acetic anhydride[7-9], the nitric acid required for the formation of the reagent being taken in excess. Excess nitric acid in concentrated sulphuric acid has been used by Campaigne and Monroe[10] to form 5-nitrothiophene-3-carboxamide **2c** from thiophene-3-carboxamide. Both processes would be inefficient if applied to a ^{15}N-labelled synthesis. However, nitration of thiophene-2-carboxamide to form solely 4-nitrothiophene-2-carboxamide **2c** in high

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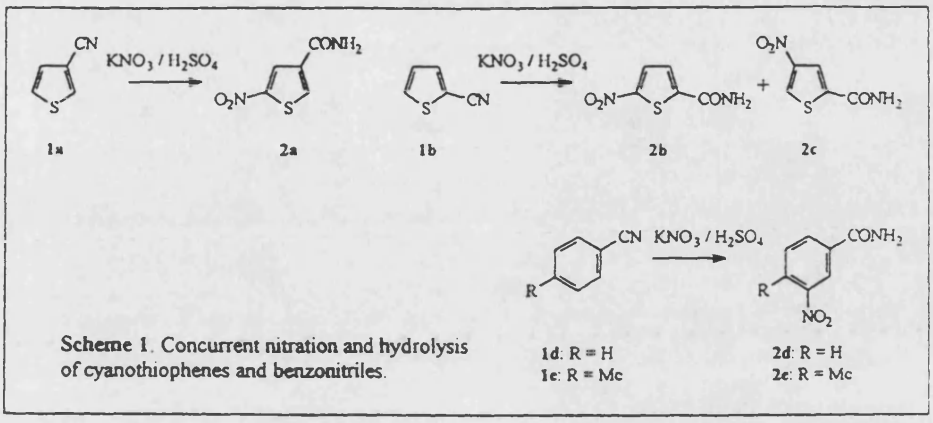


Scheme 1. Concurrent nitration and hydrolysis of cyanothiophenes and benzonitriles.

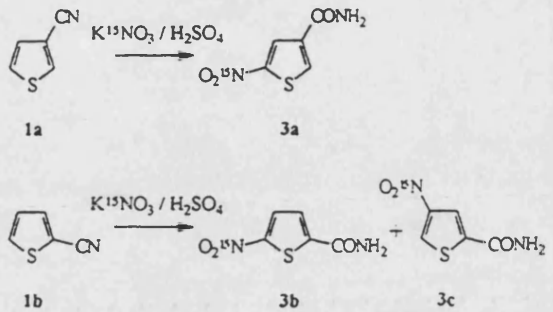
yield, using only one equivalent of potassium nitrate, has been reported by Dell'Erba *et al.*[7] Similarly, Östman[11] has described efficient nitration of 3-cyanothiophene **1a** and 2-cyanothiophene **1b** with one equivalent of nitric acid in trifluoroacetic acid, although mixtures of isomers of the corresponding cyanonitrothiophenes were produced. In this paper, we report our development of a method for direct conversion of cyano(hetero)arenes to nitro(hetero)arenecarboxamides.

Results and Discussion

The initial approach to development of potential isotopically efficient syntheses of the isomeric nitrothiophene-carboxamides was to investigate a two-step sequence from 3-cyanothiophene **1a** and 2-cyanothiophene **1b**, involving nitration, followed by hydrolysis of the nitrile. However, since the method of Östman[11] (HNO_3 / CF_3CO_2H) gives mixtures of isomers of cyanonitrothiophenes, the method of Dell'Erba[7] was applied to the cyanothiophenes **1a,b**. Unexpectedly, treatment of 3-cyanothiophene **1a** in concentrated sulphuric acid with one equivalent of potassium nitrate gave 5-nitrothiophene-3-carboxamide **2a** in 91% yield, rather than the corresponding nitrile, as shown in Scheme 1. No cyanonitrothiophenes were isolated. Clearly, the vigorously acidic conditions had been sufficient to hydrolyse the nitrile to the carboxamide. Application of this process to 2-cyanothiophene **1b** gave a mixture of 5-nitrothiophene-2-carboxamide **2b** and 4-nitrothiophene-2-carboxamide **2c** in good yield in *ca.* 4:3 ratio. These were easily separable by chromatography. No cyanonitrothiophenes were evident in the product mixture. The process was extended to the benzene series by high-yielding direct conversions of benzonitrile **1d** to 3-nitrobenzamide **2d** and 4-methylbenzonitrile **1e** to 4-methyl-3-nitrobenzamide **2e**.

For the syntheses introducing ^{15}N, potassium [^{15}N]-nitrate was prepared by neutralisation of [^{15}N]-nitric acid (95 atom %) with the calculated amount of potassium carbonate in water, followed by removal of the solvent by freeze-drying. Addition of this material to 3-cyanothiophene **1a** in concentrated sulphuric acid gave 5-[^{15}N]-nitrothiophene-3-carboxamide **3a** in excellent chemical and

isotopic yields (82%), as shown in Scheme 2. Similar [^{15}N]-nitration of **1b** gave satisfactory chemical yields of the isomeric [^{15}N]-nitro-thiophene-carboxamides **3b,c**, after chromatographic separation. The total isotopic yield for this process was 52%.



Scheme 2. Synthesis of [^{15}N]-nitrothiophene-carboxamides

The ^{15}N-labelled isotopomers **3a-c** were characterised by comparison of spectra and mpts with those of the unlabelled materials **2a-c**. In addition, ^{15}N NMR and mass spectrometry demonstrated that each product contained only one ^{15}N, as required. The ^{15}N NMR signals all appeared in the range δ 365 to δ 368, as appropriate for $Ar^{15}NO_2$. No coupling of ^{15}N to other nuclei was seen in the ^{15}N spectra, probably owing to insufficient digital resolution. However, couplings to ^{15}N were observed through ^{1}H and ^{13}C spectra. In the 5-nitrothiophene-3-carboxamide **3a**, ^{15}N coupled to protons on the thiophene ring with the three-bond coupling constant ^{3}J = 1.1 Hz. In contrast, ^{15}N-^{1}H coupling was only evident to the 3-H in **3c** and to the 4-H in **3c**, with ^{3}J = 1.1 Hz in both cases. One-bond ^{15}N-^{13}C coupling was clearly seen for all three compounds, with ^{1}J = *ca.* 20 Hz. The only three-bond ^{15}N-^{13}C coupling was in the spectrum of **3a**, between ^{15}N in the nitro group and the quaternary carbon 3-C bearing the carboxamide; ^{3}J = 3.7 Hz.

Conclusion

Direct rapid one-pot conversions of cyanothiophenes to nitrothiophene-carboxamides and of benzonitriles to 3-nitrobenzamides have been developed. In the thiophene cases, these have been extended to provide highly isotopically efficient syntheses of [^{15}N]-nitrothiophene-carboxamides. The source of ^{15}N is potassium [^{15}N]-nitrate, which is derived from the readily available and inexpensive [^{15}N]-nitric acid. This technique may have more general applications in introduction of ^{15}N in syntheses using stoichiometric amounts of [^{15}N]-reagents.

Experimental

[^{15}N]-Nitric acid (95 atom %, ca. 5 M) was purchased from MSD Isotopes. Infra-red spectra were obtained using potassium bromide discs. Jeol GX270 and EX400 instruments furnished the NMR spectra of solutions in $(CD_3)_2SO$. The ^{15}N chemical shifts are referenced externally to [^{15}N]-ammonium nitrate (2.9 M in 1.0 M aqueous hydrochloric acid: δ_N +24.90)[12]. Mass spectra were

obtained in the electron impact (EI) mode, except where noted. Melting points are uncorrected. Solvents were evaporated under reduced pressure. The chromatographic stationary phase was silica gel.

5-Nitrothiophene-3-carboxamide (2a). Potassium nitrate (1.85 g, 18.3 mmol) was added to 3-cyanothiophene **1a** (2.00 g, 18.3 mmol) in concentrated sulphuric acid (20 ml). The mixture was stirred at ambient temperature for 16 h before being poured onto ice and extracted with ethyl acetate. The extract was washed with water and with 10% aqueous sodium carbonate and was dried ($MgSO_4$). Evaporation and recrystallisation (ethanol) gave 5-nitrothiophene-3-carboxamide **2a** (2.86 g, 91%) as a pale buff solid: mp 161-162°C (lit.[13] mp 162-163°C); ν_{max} 3450, 3300, 1700, 1670 cm^{-1}; δ_H 7.63 (1 H, s, NH), 8.11 (1 H, s, NH), 8.47 (1 H, d, J = 0.8 Hz, 2-H), 8.51 (1 H, d, J = 0.8 Hz, 4-H); δ_C 128.11, 136.66, 136.90, 151.19, 161.85; *m/z* 172 (M).

5-Nitrothiophene-2-carboxamide (2b) and 4-nitrothiophene-2-carboxamide (2c). 2-Cyanothiophene **1b** was treated with potassium nitrate and concentrated sulphuric acid, as for the synthesis of **2a**. Chromatography (hexane / ethyl acetate 3:2) gave 4-nitrothiophene-2-carboxamide **2c** (41%) as a white solid: mp 151-152°C (lit.[7] mp 152-153°C); ν_{max} 3480, 1715, 1620 cm^{-1}; δ_H 7.82 (1 H, s, NH), 8.33 (1 H, s, NH), 8.42 (1 H, d, J = 1.5 Hz, 5-H), 8.94 (1 H, d, J = 1.5 Hz, 3-H); *m/z* 172 (M). From later fractions was obtained 5-nitrothiophene-2-carboxamide **2b** (32%) as a white solid: mp 191-193°C (compound reported by Occhipinti *et al.*[14] and by Johnson *et al.*[15] but no mp given); ν_{max} 3460, 1660, 1620 cm^{-1}; δ_H 7.79 (1 H, d, J = 4.4 Hz, 3-H), 7.99 (1 H, s, NH), 8.14 (1 H, d, J = 4.4 Hz, 4-H), 8.45 (1 H, s, N-H); *m/z* 172 (M).

3-Nitrobenzamide (2d). Benzonitrile **1d** was treated with potassium nitrate and concentrated sulphuric acid, as for the synthesis of **2a**, to give 3-nitrobenzamide **2d** (73%) as an pale orange solid: mp 136-138°C (lit.[16] mp 142-143°C); ν_{max} 3460, 3350, 1695, 1625 cm^{-1}; δ_H 7.75 (1 H, s, NH), 7.78 (1 H, t, J = 7.7 Hz, 5-H), 8.32 (1 H, dd, J 8.8, 1.1 Hz, 6-H), 8.37 (1 H, s, NH), 8.39 (1 H, ddd, J = 1.1, 1.5, 8.6 Hz, 4-H), 8.70 (1 H, *ca.* t, J *ca.* 1.5 Hz, 2-H); δ_C 122.24, 125.89, 130.06, 133.81, 135.77, 147.80, 165.71; *m/z* 166 (M).

4-Methyl-3-nitrobenzamide (2e). 4-Methylbenzonitrile **1e** was treated with potassium nitrate and concentrated sulphuric acid, as for the synthesis of **2a**, to give 4-methyl-3-nitrobenzamide **2e** (96%) as an off-white solid: mp 161-163°C (lit.[17] mp 168-169°C); ν_{max} 3450, 1685, 1615 cm^{-1}; δ_H 2.57 (3 H, s, Me), 7.61 (1 H, d, J = 8.1 Hz, 5-H), 7.65 (1 H, s, NH), 8.13 (1 H, dd, J = 7.7, 1.8 Hz, 6-H), 8.24 (1 H, s, NH), 8.47 (1 H, d, J = 1.8 Hz, 2-H); δ_C 19.54, 123.45, 131.89, 133.03, 133.44, 135.95, 148.84, 165.69; *m/z* (CI) 181 (M + H).

5-[^{15}N]-Nitrothiophene-3-carboxamide (3a). Potassium [^{15}N]-nitrate (79 mg, 0.77 mmol, 95 atom %) was added to 3-cyanothiophene **1a** (840 mg, 0.77 mmol) in concentrated sulphuric acid (1.0 ml). Isolation, as for the synthesis of **2a**, gave 5-[^{15}N]-nitrothiophene-3-carboxamide **3a** (110 mg, 82%) as a pale buff solid: mp 158-160°C (lit.[13] mp 162-163°C for **2a**); δ_H 7.62 (1 H, s, NH), 8.10 (1 H, s, NH), 8.46 (1 H, dd, $J_{H\text{-}H}$ = 1.8 Hz, $J_{H\text{-}N}$ = 1.1 Hz, 4-H), 8.51 (1 H, dd, $J_{H\text{-}H}$ = 1.8 Hz, $J_{H\text{-}N}$ = 1.1 Hz, 2-H); δ_C 128.05, 136.59 (d, $^3J_{C\text{-}N}$ = 3.7 Hz, 3-C), 136.85, 151.10 (d, $^1J_{C\text{-}N}$ = 20.3 Hz, 5-C), 161.79; δ_N +365.07; *m/z* (EI) 173 (M).

5-[^{15}N]-Nitrothiophene-2-carboxamide (3b) and 4-[^{15}N]-nitrothiophene-2-carboxamide (3c). Potassium [^{15}N]-nitrate (280 mg, 2.75 mmol, 95 atom %) was added to 2-cyanothiophene 1b (840 mg, 0.77 mmol) in concentrated sulphuric acid (1.0 ml). Isolation and purification, as for the synthesis of **2b,c**, gave 4-[^{15}N]-nitrothiophene-2-carboxamide **3c** (180 mg, 38%) as a pale buff solid mp 151-152°C (lit.[7] mp 152-153°C for **2c**); δ_H 7.80 (1 H, s, NH), 8.32 (1 H, s, NH), 8.40 (1 H, d, J = 1.5 Hz, 5-H), 8.93 (1 H, dd, $J_{H\text{-}H}$ = 1.5 Hz, $J_{H\text{-}N}$ = 1.1 Hz, 3-H); δ_C 122.49, 133.16, 141.48, 147.07 (d, $^1J_{C\text{-}N}$ = 18.4 Hz, 4-C), 161.32; δ_N +367.48; *m/z* (EI) 173 (M). Further elution gave 5-[^{15}N]-nitrothiophene-2-carboxamide **3b** (69 mg, 14%) as an off-white solid: mp 188-190°C, δ_H 7.78 (1 H, d, J = 4.4 Hz, 3-H), 7.98 (1 H, s, NH), 8.14 (1 H, dd, $J_{H\text{-}H}$ = 4.4 Hz, $J_{H\text{-}N}$ = 1.1 Hz, 4-H), 8.44 (1 H, s, NH); δ_C 127.87 (d, $^3J_{C\text{-}N}$ = 3.7 Hz, 2-C), 130.27, 146.93, 152.93 (d, $^1J_{C\text{-}N}$ = *ca.* 20 Hz, 5-C), 161.30; δ_N +367.52; *m/z* (EI) 173 (M).

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Uptake and Retention of Nitroimidazole-Carboranes for Boron Neutron Capture Therapy in Experimental Murine Tumours: Detection by ^{11}B Magnetic Resonance Spectroscopy

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Uptake and retention of nitroimidazole-carboranes designed for boron neutron capture therapy in experimental murine tumours: detection by ^{11}B magnetic resonance spectroscopy

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Abstract. Two novel nitroimidazole-carboranes were examined for their uptake and retention in two experimental murine solid tumours and in some normal tissues, using *in vivo* ^{11}B magnetic resonance spectroscopy. The compounds were injected i.p. at 0.8mmol/kg into mice bearing either the SCCVII/Ha squamous cell carcinoma or KHT sarcoma implanted intradermally on the mouse back. Boron from a polyether-isoxazole linked nitroimidazole-carborane (compound 1) was detectable in both SCCVII/Ha and KHT tumours at 3 and 7 h after injection. The signal from the liver at these times was greater than that from the tumour but only a weak signal was obtained from the brain. At 24 h after injection the tumour signal was still present, as was that from the liver, which appeared to have increased over that for the earlier times. Signal from the brain had disappeared by 24 h. Boron from a polyether-carbamate linked nitroimidazole-carborane (compound 2) was also detectable in both tumours at all times tested, and again was present in the liver. In addition, the ^{11}B signal was detectable from the mouse brain, at early times, but was undetectable at 24 h. These preliminary data indicate that nitroimidazole-carboranes are taken up and retained in experimental murine tumours in sufficient amounts to be detectable by *in vivo* ^{11}B MRS and further that at 24 h after treatment there is differential retention between tumours and the brain.

1. Introduction

Recently there has been a resurgent interest in the use of boron neutron capture therapy (BNCT) for the treatment of certain types of cancer, including glioma and melanoma (Barth *et al.* 1990, 1992). Studies from Japan indicate promising survival rates for patients treated with BNCT (Hatanaka and Nakagawa 1994). Considerable research is ongoing to design tumour-targeted neutron capture agents which would improve the efficacy of this form of treatment.

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BNCT involves the interaction of thermal neutrons with the ^{10}B nucleus, as follows:

$$^{10}B + n_{th} \rightarrow [^{11}B] \rightarrow {}^{7}Li + {}^{4}He + 2{\cdot}31MeV.$$

The highly reactive α particle (^4He nucleus) produced in this reaction can exert its damaging effect over a distance of approximately one cell diameter. This implies that damage from BNCT will be localized largely to cells containing ^{10}B.

The failure of BNCT in early studies has been attributed to two major problems; (1) the inability to attain adequate concentrations of ^{10}B in tumours, and (2) the lack of selectivity of boron containing compounds to tumours, leading to normal tissue damage. Recent studies have used a range of compounds to target ^{10}B to tumours (Hawthorne 1993 for review), including boron-substituted nucleosides (Tjarks *et al.* 1992, Goudgaon 1994), amino acids (Wyzlic and Soloway 1992, Coderre *et al.* 1994, Wyzlic *et al.* 1994) and porphyrins (Miura *et al.* 1992).

A novel approach to the selective uptake of compounds into tumours is based upon the knowledge that most solid tumours contain regions of hypoxia, which may be targeted by bioreductive cytotoxic agents, such as nitroimidazoles, which can selectively bind to hypoxic cells (Kennedy *et al.* 1980, Stratford 1992, Adams *et al.* 1984, Keyes *et al.* 1985). It follows that the attachment of boron-containing compounds to such nitroimidazoles may result in hypoxia-mediated tumour localization of the boron moiety, which could therefore result in more tumour selective application of BNCT. Compounds of this type have been synthesized (Scobie and Threadgill 1994a,b, Wilbur *et al.* 1994, Swenson *et al.* 1996). The recent study by Swenson *et al.* (1996) has demonstrated the ability of a boronated nitroimidazole to sensitize tumour cells *in vitro* to neutron irradiation.

In the present study, two compounds linking the stable icosahedral $C_2B_{10}H_{12}$ carborane to

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2-nitroimidazole (Scobie and Threadgill 1994a,b), were administered to mice bearing experimental murine solid tumours. *In vivo* ^{11}B magnetic resonance spectroscopy was used to examine the uptake and retention of these compounds in the tumours and in some normal mouse tissues.

2. Materials and methods

2.1. *Mice and tumours*

The SCCVII/Ha squamous cell carcinoma and the KHT sarcoma were used for the study. SCCVII/Ha was maintained as described by Twentyman *et al.* (1980). KHT was maintained by continuous passage *in vivo*. Tumours for experiment were implanted by injection of 2×10^5 tumour cells in 0·05ml culture medium, intradermally on the back of C_3H/He male or female mice, 2cm from the tail base, and were used for experiment 10–14 days later at a volume of 200–500mm^3. Mice were maintained and experiments conducted in accordance with the Animals (Scientific Procedures) Act 1986.

2.2. *Anaesthesia*

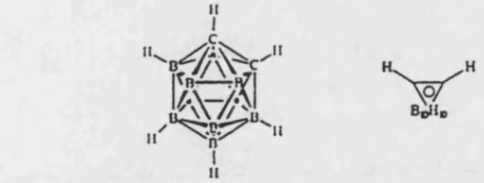
Mice for experiments were anaesthetized using Hypnorm/Hypnovel. (Hypnorm®: Fentanyl citrate 0·315mg/ml; fluanisone 10mg/ml, Janssen Pharmaceutica, BV Tilburg, The Netherlands. Hypnovel®: Midazolam 5mg/ml, Roche Products, Welwyn Garden City, UK). The anaesthetic mixture was Hypnorm : Hypnovel : water (1 : 1 : 2) and was injected i.p. at 0·1ml/mouse.

2.3. *Nitroimidazole-carboranes*

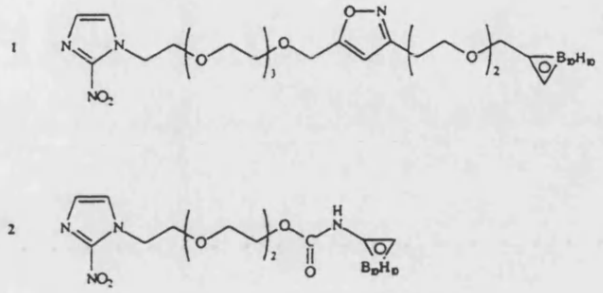
Compounds were synthesized as described previously (Scobie and Threadgill 1994a,b). Representations of the carborane structure are given in Figure 1A. The structures of compounds **1** and **2** are given in Figure 1B.

Both compounds were dissolved in DMSO at 200mg/ml. Subsequent dilutions were in peanut oil which, after brief sonication, were stable enough to permit injection.

Both compounds were injected i.p. at a single test dose of 0·81mmol/kg (500mg/kg for compound 1; 350mg/kg for compound 2) at 0·01ml/g mouse body weight, and were well tolerated.



A



B

Figure 1. (A) Representations of the icosahedral carborane. (B) Structures of polyether-isoxazole linked nitroimidazole-carborane, 1, and polyether-carbamate linked nitroimidazole-carborane 2.

2.4. *^{11}B magnetic resonance spectroscopy*

2.4.1. *In vitro.* Spectra of the nitroimidazole-carboranes were obtained using a 9.4 tesla vertical bore magnet with a Jeol EX400 spectrometer, at a tuning frequency of 128·33Hz, with and without broadband proton decoupling. Samples were at a concentration of 100mM in $CDCl_3$ in a spinning 5mm tube. $BF_3.Et_2O$ was used as external reference. A spectrum of compound **1** was also obtained using the 4.7 tesla system as described below, with the sample in a 5ml glass round-bottomed flask.

2.4.2. *In vivo.* Spectra of the nitroimidazole-carboranes were obtained *in vivo* using a 4.7 tesla 30cm horizontal bore magnet (Oxford Instruments) with a SISCO 200 spectrometer. A 1cm triple turn surface coil, tuned to 64·18Hz was placed over the tumour, liver or mouse brain. A 12μs pulse width with a 1s delay was used and each spectrum comprised 1024 repetitions, giving a total acquisition time of 17min. 100Hz line broadening was applied to each spectrum.

3. Results

^{11}B MR spectra were obtained from the nitroimidazole-carboranes *in vitro* using a 9·4 T magnet. Spectra for compound 1 are given in Figure 2A and showed five peaks in the range δ_B −4·5 to −15·0ppm ($^1J_{B-H}$ = 150Hz) relative to the external standard. The complexity of the spectra reflect the different environments of the boron nuclei within the carborane structure (Scobie and Threadgill 1994b).

In addition, for direct comparison with the *in vivo* spectra, a spectrum from compound 1 *in vitro* was obtained using the 4.7 tesla system, and is given in Figure 2B. The presence of a 'hump' in the *in vitro* spectra was due to signal from the borosilicate glass tubes in which the compounds were contained.

^{11}B MR spectra were also obtained *in vivo* in SCCVII/Ha and KHT tumours at various times after i.p. injections of the two compounds. Although the absence of internal standards precluded the absolute determination of concentrations of the compounds from the MR spectra, a semiquantitative analysis was made by determination of peak areas, which were expressed as a ratio for tumour compared to each normal tissue.

Figure 3 gives representative *in vivo* ^{11}B MR spectra from (A) SCCVII/Ha tumour, together with (B) the mouse liver and (C) brain at 3, 7 and 24 h after i.p. injection of compound 1 at 0·81mmol/kg. The ^{11}B signal was detectable in the tumour at 3 and 7 h after injection, which was sustained until 24 h. Signal from the brain was also detectable at early times after injection, but was lost by 24 h. The tumour/brain peak ratio was 2–3 at the early times after injection of compound 1. By contrast, a strong signal was detected from the liver at 3 and 7 h after injection (liver/tumour peak ratio = 5–6), which had increased substantially by 24 h (liver/tumour peak ratio = 20). However, this signal was lost by 4 days (spectrum not shown). Similar results were observed in three other SCCVII/Ha tumour-bearing mice, and in two KHT tumour-bearing mice.

In order to determine whether the large signal from the liver was due to residual compound 1 in the peritoneal cavity, compound 1 was given orally to two mice. The boron signal from the liver after oral administration of compound 1 was similar to that for i.p. injection (spectrum not shown).

Figure 4 gives representative *in vivo* ^{11}B MR spectra from (A) KHT tumour, together with (B)

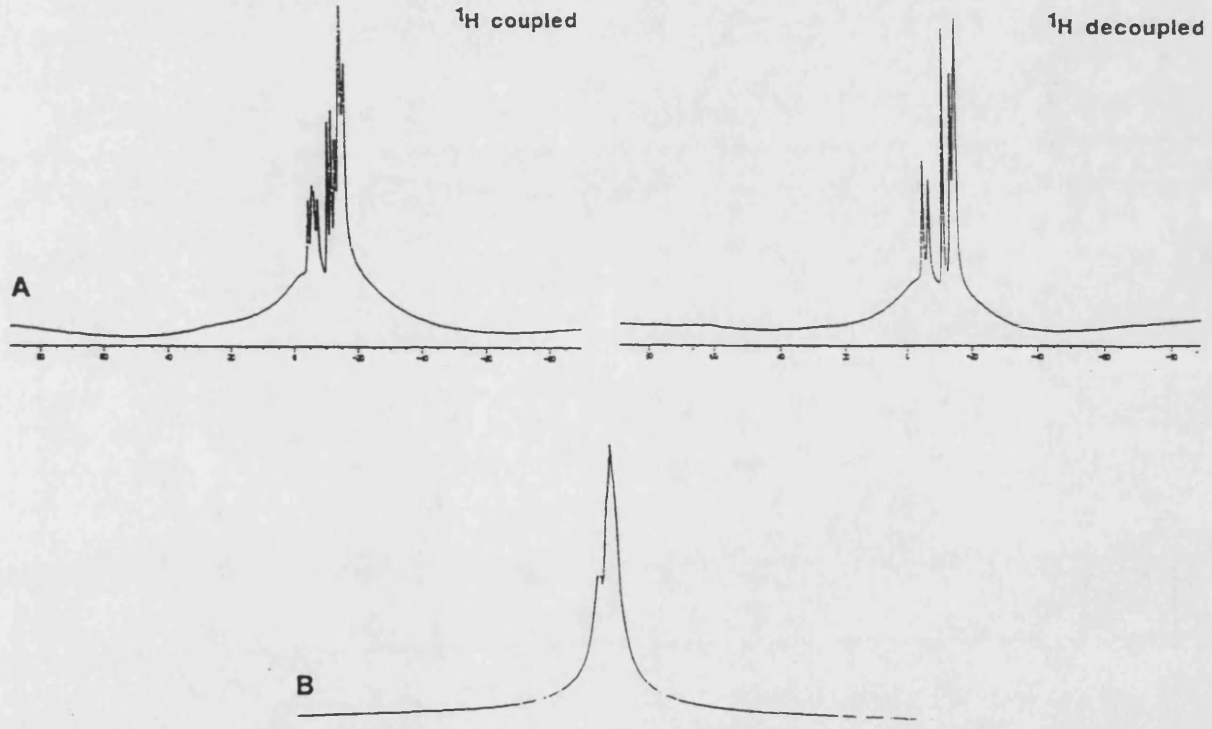


Figure 2. (A) *In vitro* ^{11}B MR spectrum of 100 mM solution of compound 1 obtained from a 9.4 tesla system. (B) *In vitro* ^{11}B MR spectrum of 200 mg/ml solution of compound 1 obtained from a 4.7 tesla system. Details are given in §2.

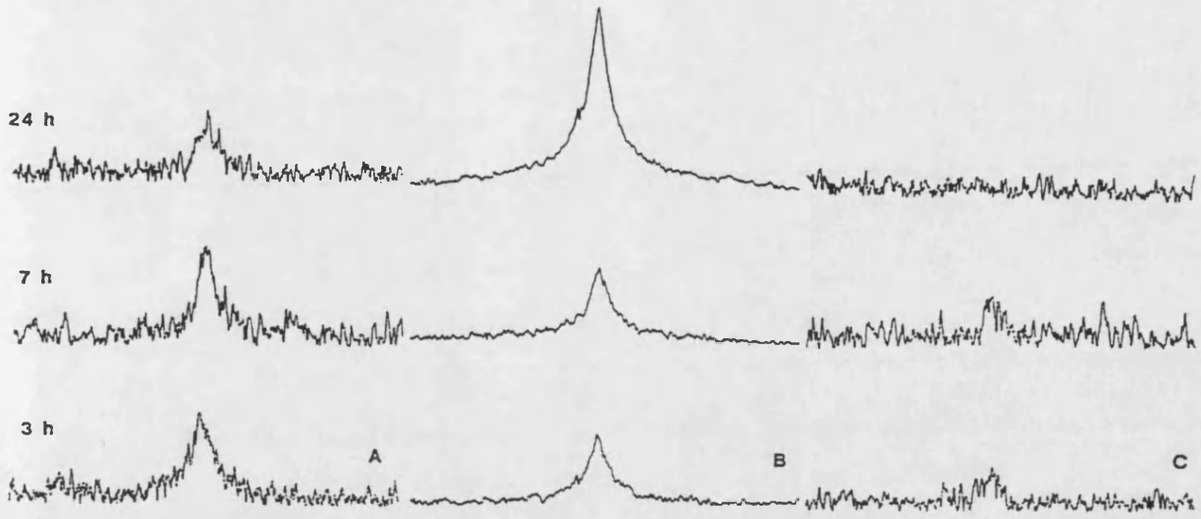


Figure 3. *In vivo* ^{11}B MR spectra from (A) SCCVII/Ha tumour, (B) liver and (C) brain in C_3H mouse, 3, 7 and 24 h after i.p.injection of 0·81 mmol/kg of compound 1. The vertical scale for the liver spectra is one-fifth of that for the spectra from tumour and brain.

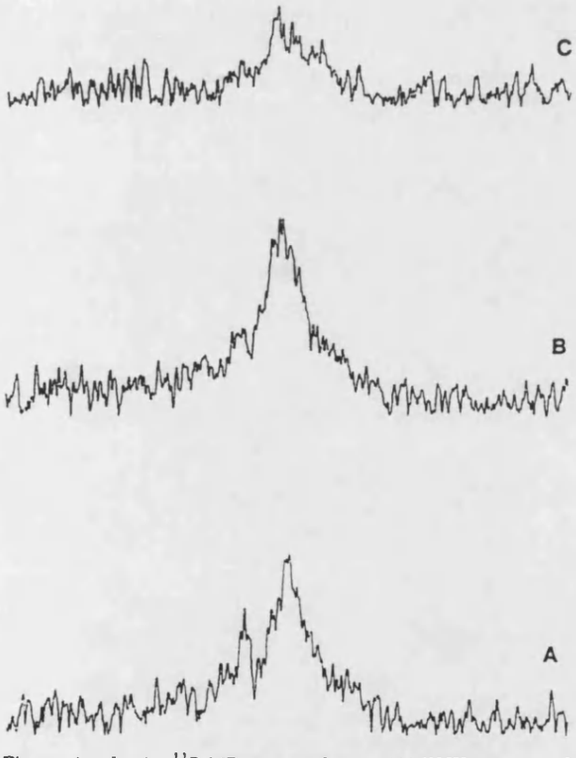


Figure 4. *In vivo* ^{11}B MR spectra from a (A) KHT tumour, (B) liver and (C) brain in C_3H mouse, 4 h after i.p. injection of 0·81 mmol/kg of compound 2. The vertical scale is the same for all spectra.

the mouse liver and (C) brain at 4 h after i.p. injection of compound 2 at 0·81 mmol/kg. Again, the ^{11}B signal was detectable from the tumour and brain, and the tumour/brain peak ratio was 3. The signal from the liver at this time appeared to be much less than that observed for compound 1 (liver/tumour peak ratio = 1). At 24 h after injection, signal was detectable from the tumour and liver, but not from the brain (spectra not shown). Similar results were obtained in one other KHT tumour-bearing mouse and in two SCCVII/Ha tumour-bearing mice.

4. Discussion

The results presented here are the outcome of a preliminary study designed to ask whether the attachment of boron containing compounds to bioreductive agents was a feasible means of targetting boron to solid tumours and whether such compounds were detectable by *in vivo* ^{11}B MRS.

Two nitroimidazole-carboranes were examined, in two murine transplantable tumours, and the results indicate that both compounds were detectable in these tumours by *in vivo* ^{11}B MRS. It is well established that *in vivo* MRS can in general, provide a useful, non-invasive method for the detection of metabolites and drugs in tissues. ^{11}B MRS and MRI have been used previously to detect boron containing compounds *in vivo* (Kabalka *et al.* 1988, Bendel *et al.* 1990, Kabalka *et al.* 1991), and

^{11}B MRS has been used successfully in this study. It is important to emphasize that these compounds are being taken up into the tumours or tissues in sufficient quantities to permit their detection by this method.

Although the detection and analysis of the nitroimidazole-carboranes *in vivo* by ^{11}B MRS is only semiquantitative, the spectra give some indication of the relative levels of these compounds in tumours compared with normal tissues. The ^{11}B MR spectra indicate higher levels of the compounds in the liver, compared with tumour. This phenomenon has been observed with other nitroimidazoles, such as misonidazole (Garrecht and Chapman 1983). This apparent non-selectivity of uptake of these compounds may not be a problem in a possible BNCT strategy where application of radiation is localized. However, the results do suggest some selectivity of uptake of the componds in the tumours compared with the brain. Clearly, quantification of levels on a tissue-weight basis would be necessary before definite conclusions could be made and the normal tissue toxicity of these compounds themselves must be an important consideration. Important further experiments would examine the uptake and retention of compounds of this type in tumours located in the brain. The apparent weaker boron signals from the brain may be indicative of low levels of neurotoxicity for the two compounds.

In the experimental murine tumour systems, the compounds were well tolerated by the mice at the dose of 0·81mmol/kg. The use of higher doses was limited by the concentration of DMSO in the solvent. Thus, improved aqueous solubility would be an advantage, and this will be the focus of future synthetic work.

The design of the nitroimidazole-carboranes was based upon the hypoxia-selectivity of the bioreductive part of the molecule, in order to retain boron in the tumour cells. The presence of hypoxia in experimental murine solid tumours has been demonstrated (Hoeckel *et al.* 1991, Okunieff *et al.* 1993), and the presence of the nitroimidazole-carboranes in two tumours of this type up to 24 h after administration, strongly suggests that hypoxia induced binding may be a mechanism of uptake of these compounds. However, the apparent higher levels of these compounds in the liver suggest that hypoxia related uptake and retention of these agents may not be the only mechanism occurring. Nevertheless, the detection of these compounds in solid tumours suggests that hypoxia may be playing a role in their uptake, and this may be enhanced further by selectively increasing hypoxia with agents which alter tumour blood flow and metabolism (Stratford *et al.* 1994). This could be achieved for example, with agents such as inhibitors of nitric oxide synthase, which induce hypoxia in these tumours at doses which have no significant effect on normal tissue metabolism (Wood *et al.* 1994).

The results presented here describe a novel approach to the problem of achieving sufficiently high levels of intracellular boron in solid tumours for BNCT, using compounds specifically designed to exploit the presence of hypoxia known to exist in most solid tumours.

We have demonstrated that two such compounds can be taken up into experimental tumours in sufficient amounts to be detectable by *in vivo* ^{11}B MRS. While considerable work is needed to optimize the uptake and selectivity of these compounds, the preliminary data show this to be a very promising approach for BNCT.

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PUBLICATION 75

Synthesis of a Series of Trifluoromethylazoles and Determination of pK_a of Acidic and Basic Trifluoromethyl Heterocycles by ^{19}F NMR Spectroscopy

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Synthesis of a series of trifluoromethylazoles and determination of pK_a of acidic and basic trifluoromethyl heterocycles by ^{19}F NMR spectroscopy

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Trifluoroacetylation at the 5-position of 3,4-dihydro-2*H*-pyran and the 3-position of 4,5-dihydrofuran, followed by treatment with hydrazine, gave 3-(3-trifluoromethyl-1*H*-pyrazol-4-yl)propanol and 2-(3-trifluoromethyl-1*H*-pyrazol-4-yl)ethanol, respectively. In the latter case, an intermediate dimer was isolated. Isomeric 2-(3-trifluoromethyl-1*H*-pyrazol-5-yl)ethanol was formed by reaction of hydrazine with 6-benzyloxy-1,1,1-trifluorohex-3-yn-2-one and deprotection. Reaction of 3-benzyloxypropylamine with 2,5-bis(trifluoromethyl)-1,3,4-oxadiazole, followed by deprotection, afforded 3-[3,5-bis(trifluoromethyl)-4*H*-1,2,4-triazol-4-yl]propanol. A series of 2-trifluoromethyl-1*H*-benzimidazoles and 2-trifluoromethyl-3*H*-imidazopyridines were prepared by condensation of the appropriate *ortho*-arenediamine with trifluoroacetic acid. Analysis of the ^{19}F NMR spectra of the trifluoromethylazoles and of 3-trifluoromethylpyridine in aqueous solution at different pHs enabled determination of pK_a values. All the compounds evaluated had one or more pK_a between 1 and 13, except the triazole. Several compounds were identified as having potential use in measuring pH in biological media by ^{19}F NMR spectroscopy.

A large number of pyrazoles and derived heterocycles have found use[1] as therapeutic agents and as agrochemicals. Trifluoromethyl heterocycles are also of considerable interest in these areas[2] on account of the acid-strengthening/base-weakening electronic effects of incorporation of the trifluoromethyl group and in view of the increased lipophilicity of compounds bearing this functionality. As part of a programme of synthesis and evaluation of trifluoromethyl heterocycles for medicinal and pH sensor applications,[3,4] we required pyrazoles and 1,2,4-triazoles with one or more trifluoromethyl groups on the heterocycle, and also carrying an ω-hydroxyalkyl function for later attachment to various moieties, such as 2-nitroimidazole,[5] to permit biological targeting to specific tissue or cell types.

Of the methods available for the synthesis of N-unsubstituted pyrazoles, those involving condensation of hydrazine with 1,3-diketones and with α,β-acetylenic ketones are among the more widely used.[6] Clearly, the range of pyrazoles that can be prepared by both methods is limited to those where the substrate is readily synthetically accessible and, in the latter case, to 4-unsubstituted pyrazoles. We[4] and others[7] have recently reported the synthesis of 3,5-bis(trifluoromethyl)-pyrazole from 1,1,1,5,5,5-hexafluoropentane-2,4-dione but all attempts to alkylate this 1,3-diketone at C-3, leading to 4-substituted 3,5-bis(trifluoromethyl)pyrazoles, were unsuccessful. For our series of target pyrazoles, with ω-hydroxyalkyl substituents, there is an additional requirement for introduction or appropriate protection of the hydroxy group.

In planning the synthesis of 3-(3-trifluoromethyl-1*H*-pyrazol-4-yl)propanol **3** and 2-(3-trifluoromethyl-1*H*-pyrazol-4-yl)ethanol **8**, where the ω-hydroxyalkyl group is at the 4-position of the pyrazole, we recognised that the trifluoroacetyldihydropyran **2** and the trifluoroacetyldihydrofuran **6**, respectively, contain the correct carbon skeleton and a masked 1,3-diketone for condensation with hydrazine. Furthermore, this condensation would reveal the required ω-hydroxyalkyl function as a leaving group, thus obviating the need for earlier introduction and possible protection of the hydroxy group.

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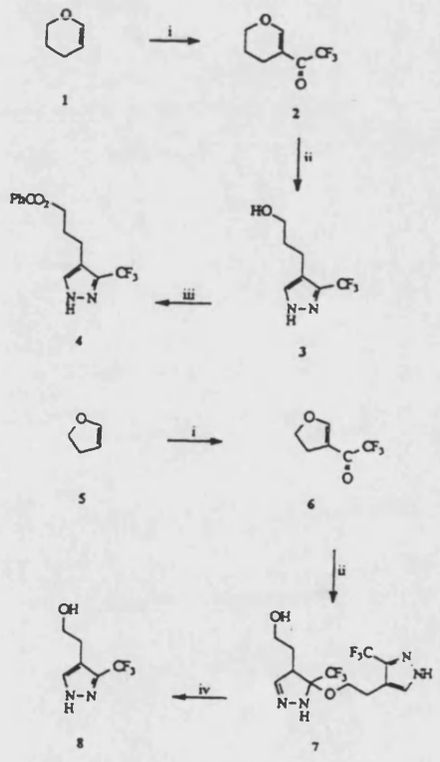
Dihydropyran **1** and dihydrofuran **5** were trifluoroacetylated using trifluoroacetic anhydride and pyridine in dichloromethane, as shown in Scheme 1. The yields in these first steps were



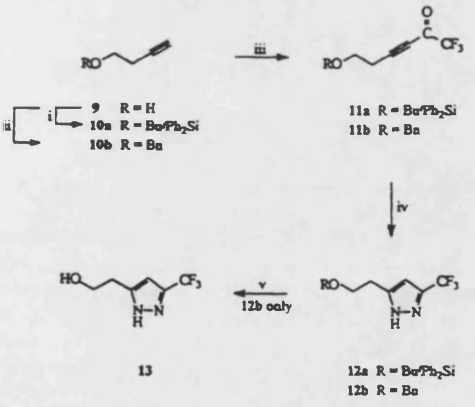
Scheme 1 *Reagents*: i, $(CF_3CO)_2O$, pyridine, CH_2Cl_2; ii, N_2H_4, EtOH; iii, BzCl; iv, HCl, EtOH

notably higher than those reported by Hojo *et al.*[8] for the same procedure. Treatment of **2** with hydrazine in boiling ethanol effected the condensation with concomitant exposure of the

3-hydroxypropyl group, as predicted. The trifluoromethylpyrazolylpropanol **3** was isolated in 96% overall yield from **1**. To ensure that the ω-hydroxy group could be derivatised without interference from the pyrazole, the corresponding benzoate ester **4** was formed in the usual way. Since this work was carried out, Tang and Hu[9] have reported the synthesis of **3** by addition of the expensive iodopentafluoroethane across the enol ether of **1**, in the presence of sodium dithionite, and treatment of the pentafluoroethyltetrahydropyran with hydrazine.

In contrast, treatment of the five-membered ring homologue **6** with hydrazine did not give the expected pyrazolylethanol **8** directly. Under essentially the same conditions as above, a virtually quantitative yield of the dimer **7** was formed. In this dimer, one molecule of the fully condensed aromatic pyrazolylethanol has intercepted another molecule where the elimination of water from the intermediate is not complete, although the detailed mechanism of the formation of **7** is not clear. It is particularly noteworthy that no material could be isolated where solvent ethanol, rather than the pyrazolylethanol, has reacted as the incoming nucleophile, despite the much higher concentration of the former. The dimer **7** readily formed the target pyrazolylethanol **8** upon reflux in the presence of a trace of acid. In a control experiment, it was demonstrated that **8** was not converted to the dimer **7** under the condensation conditions, indicating that **7** had not been formed from two molecules of **8**.

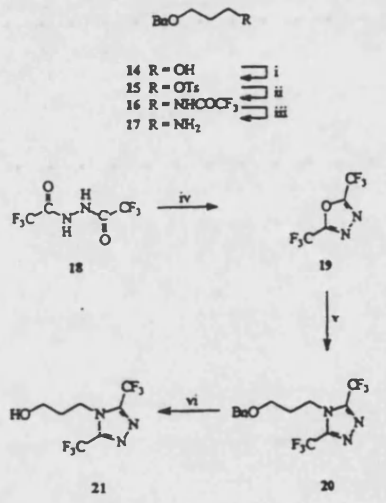
In the approach to the 3,5-disubstituted pyrazole **13**, ring formation from hydrazine and an α,β-acetylenic ketone was investigated, as shown in Scheme 2. In this case, it was not



Scheme 2 *Reagents:* i, Bu^tPh_2SiCl, imidazole, DMF; ii, BnBr, NaH, DMF; iii, BuLi, $CF_3CO_2CH_2CF_3$, THF; iv, N_2H_4, EtOH; v, H_2, Pd–C, $HClO_4$, MeOH

possible to design a process in which the hydroxyalkyl group was revealed during the condensation and it was necessary to assemble an appropriate trifluoromethyl ketone containing the alcohol, possibly in a protected form. But-3-ynol **9** was protected as its *tert*-butyldiphenylsilyl ether **10**, essentially by the method of Delorme *et al.*[10] This allowed formation of the acetylenic carbanion by treatment with butyllithium at low temperature. Addition of trifluoroethyl trifluoroacetate as the electrophile afforded the *tert*-butyldiphenylsilyl-protected acetylenic trifluoromethyl ketone **11a** in 95% yield. Other electrophilic trifluoroacetylating agents, such as trifluoroacetic anhydride, ethyl trifluoroacetate and ethyl trifluorothioacetate, were considerably less effective. Condensation of this ketone **11a** with hydrazine formed the *tert*-butyldiphenylsilyl-protected pyrazol-5-ylethanol **12a** in virtually quantitative yield. Unexpectedly, it proved impossible to remove the silyl protecting group with fluoride using conditions under which the product pyrazolylethanol **13** could be isolated. Repetition of the sequence using *O*-benzyl protection was more successful. The alcohol **9** was benzylated, essentially by the method of Johnson *et al.*,[11] to give the ether **10b**. As before, formation of the acetylenic anion and trifluoroacetylation (trifluoroethyl trifluoroacetate) gave the ketone **11b** in very high yield. Condensation with hydrazine in boiling ethanol afforded the protected 3,5-disubstituted pyrazole **12b**. Now, exposure of the hydroxy group was effected by hydrogenation under acidic conditions to afford the target trifluoromethylpyrazolylethanol **13** with the substituents in the desired 3,5-arrangement.

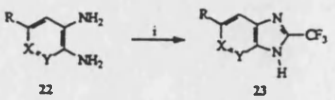
The most common routes for preparation of 1,2,4-triazoles are thermal condensation of an acylhydrazide with a (thio)-amide (the Pellizzari reaction)[12] and condensation of a diacylamine with hydrazine (the Einhorn–Brunner reaction).[13] However, the symmetry of the target compound, the 3,5-bis(trifluoromethyl)triazolylpropanol **21**, and the need for a functionalised substituent at the 4-position, suggested that condensation of an appropriately protected ω-hydroxyalkyl primary amine with an *N*,*N*′-diacylhydrazine, or its equivalent, would be a more efficient route. Scheme 3 shows the route



Scheme 3 *Reagents:* i, TsCl, KOH, Et_2O; ii, CF_3CONH_2, KOBu′, THF, NaI; iii, NaOH, MeOH; iv, P_2O_5, heat; v, **17**, MeOH; vi, H_2, Pd–C, $HClO_4$, EtOH

adopted. 3-Benzyloxypropylamine **17** was synthesised in three steps from the commercially available mono-*O*-benzyl protected propane-1,3-diol **14**. Activation of the alcohol as the toluene-*p*-sulfonate **15** was achieved using toluene-*p*-sulfonyl chloride (TsCl) in the presence of powdered potassium hydroxide. Substitution with the anion derived from trifluoroacetamide served to introduce the nitrogen atom, giving the amide **16**. Selective hydrolytic deprotection under basic conditions afforded the required *O*-benzyl-protected hydroxypropylamine **17** in 58% overall yield from **14**. In a modification of the technique used by Reitz and Finkes,[14] 1,2-bis(trifluoroacetyl)hydrazine **18** was cyclised to form the oxadiazole **19** in good yield by dehydration with phosphorus pentoxide at high temperature. Although the condensation of this normally reactive heterocycle **19** with the amine **17** proceeded only in moderate yield to form **20**, subsequent deprotection by hydrogenolysis under acidic conditions was efficient, affording the target 3-[3,5-bis(trifluoromethyl)-4*H*-1,2,4-triazol-4-yl]propanol **21** in 39% yield from the amine **17** and in 23% overall yield in five steps from **14**.

Scheme 4 shows the preparative route developed for synthesis of the 2-trifluoromethylimidazopyridines **23a,b** and the 2-trifluoromethylbenzimidazoles **23c,d**. The former pair of compounds were selected as being potentially water-soluble. The benzimidazoles were designed to carry two inequivalent sets of



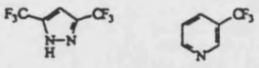
22 23

a R = H, X = N, Y = CH
b R = H, X = CH, Y = N
c R = F, X = Y = CH
d R = F_3C, X = Y = CH

Scheme 4 *Reagent:* i, CF_3CO_2H

fluorine atoms with different sensitivities of chemical shift to pH. In benzimidazole **23d**, the chemical shift of the 5-trifluoromethyl group should be sufficiently insensitive to pH for it to act as an internal chemical shift standard. Prolonged treatment of the pyridinediamines **22a,b** with boiling trifluoroacetic acid gave mixtures of regioisomeric acylaminopyridines, along with small amounts of the cyclised products **23a,b**, as shown by ^1H and ^{19}F NMR spectroscopy. Cyclisation was completed at 200 °C in a Kugelrohr apparatus, with sublimation of the target imidazopyridines **23a,b**. The benzimidazoles **23c,d** were formed by similar condensations, also in excellent yields.

The effect of pH on the ^{19}F NMR chemical shift (δ_F) of the trifluoromethyl heterocycles **3**, **8**, **13**, **21**, **23a,b**, **24** and **25** was then measured with the aim of measuring pK_a values and identifying compounds in which δ_F was highly sensitive to pH in the physiological range. Compound **24** was available in the laboratory[4] and compound **25** was obtained commercially. The benzimidazoles **23c,d** were not sufficiently soluble in aqueous media to permit these measurements to be made.



24 **25**

Pyrazole has an acidic pK_a = 14.18, as reported by Elguero *et al.*[15] who used UV spectroscopic methods. This group also studied the acid-strengthening effects of electron-withdrawing groups on pyrazoles, with 3-trifluoromethyl-5-methylpyrazole having pK_a = 12.33, while the second trifluoromethyl group, as in 3,5-bis(trifluoromethyl)pyrazole **24**, increased the acidity even more markedly (pK_a = 7.51). Similar but more pronounced effects are seen for the sequential incorporation of nitro groups.[16] Fig. 1(*a*) shows the δ_F–pH relationship for 3-(3-trifluoromethyl-1*H*-pyrazol-4-yl)propanol **3**, while Fig. 1(*b*) shows that for the lower homologue, 2-(3-trifluoromethyl-1*H*-pyrazol-4-yl)ethanol **8**. As expected, these compounds have very similar pK_a values (12.1 and 12.0, respectively). Moving the ω-hydroxyalkyl substituent to the 5-position, as in 2-(3-trifluoromethyl-1*H*-pyrazol-5-yl)ethanol **13**, had a slight acid-weakening effect, giving pK_a = 12.8. For the 3,5-bis(trifluoromethyl)-1*H*-pyrazole **24** [Fig. 1(*d*)], the pK_a was determined to be 7.55, closely in agreement with the reported value.[15] These results place the mono(trifluoromethyl)pyrazoles well outside the useful range for measurement of pH in biological systems but the pK_a of **24** is highly appropriate. All four trifluoromethylpyrazoles **3**, **8**, **13** and **24** gave simple sigmoidal curves with good sensitivity of chemical shift to changes in pH, with $\Delta\delta_F$ (the total changes in chemical shift across the whole pH range) being in the range 2.09 to 2.33 ppm for the mono(trifluoromethyl)pyrazoles and 1.47 ppm for the bis(trifluoromethyl)-pyrazole. As expected, formation of the anions at higher pHs caused the signals to move upfield.

In contrast, the ^{19}F chemical shift of the analogous 3,5-bis(trifluoromethyl)-4*H*-1,2,4-triazole **21** was virtually independent of pH in the range pH 0.0 to 9.5, as shown in Fig. 2. As this compound cannot be a heterocyclic acid, unlike the pyrazoles, no studies were made under more alkaline conditions. Thus, either the corresponding triazolium cation has δ_F identical to that of the unionised triazole or, more likely, the pK_a of the compound is <0.0.

The relationships of δ_F to solution pH for the isomeric 2-trifluoromethylimidazopyridines **23a,b** both approximated closely to double sigmoidal curves (Fig. 3), indicating two ionisation events for each compound with different pK_a values. The principal ionisation event for 2-trifluoromethyl-3*H*-imidazo[4,5-*c*]pyridine **23a**, loss of the imidazole N–H proton with the neutral molecule acting as an acid, occurs at pK_a = 8.78. There is also an ionisation event at pK_a = *ca.* 3.5, probably corresponding to protonation of the neutral molecule at the pyridine nitrogen. The former event causes greater change in chemical shift than the latter ($\Delta\delta_F$ 1.0 and $\Delta\delta_F$ 0.1, respectively). The curve for 2-trifluoromethyl-3*H*-imidazo[4,5-*b*]pyridine **23b** is similar in shape. The pK_a values are 6.92 and *ca.* 2.2, representing the acidic and basic ionisations, respectively. The fact that the principal pK_a of this compound lies well within the physiological pH range makes it an attractive candidate lead compound for measurement of pH in biological systems, although the $\Delta\delta_F$ is relatively small (0.55 ppm). For comparison, the analogous 2-trifluoromethylbenzimidazole has been reported[17] to have pK_a = 8.13, as measured spectroscopically, but it was too insoluble to allow investigation by ^{19}F NMR spectroscopy.

Finally, commercially available 3-trifluoromethylpyridine **25** was investigated. As shown in Fig. 4, this compound gave a simple sigmoidal curve with pK_a measured as 2.67 and $\Delta\delta_F$ = 1.0 ppm. Clearly, this represents protonation at the pyridine nitrogen but the basicity of **25** is much reduced by incorporation of the electron-withdrawing trifluoromethyl group (*cf.* 3-methylpyridine pK_a = 5.68)[18] which is apparently similar in effect to a chlorine atom (3-chloropyridine pK_a = 2.84).[18]

In conclusion, syntheses of four trifluoromethylpyrazoles, one trifluoromethyl-1,2,4-triazole, two trifluoromethylbenzimidazoles and two trifluoromethylimidazopyridines have been achieved. Studies of the effect of solution pH on the ^{19}F NMR chemical shift have revealed sensitivity of δ_F to the state of ionisation of the trifluoromethyl heterocycles and hence to the solution pH. Three compounds, **23a,b** and **24**, have been identified as having pK_a close to the physiological range and therefore as having potential for use in a non-invasive method of measuring pH in biological systems by ^{19}F NMR spectroscopy. In all the compounds examined, the trifluoromethyl group acts in two roles; reporter of the state of ionisation and acid-strengthener/base-weakener. Further development of the lead compounds will involve incorporation of appropriate functional groups to permit attachment of biological targeting moieties and to 'fine-tune' the pK_a.

Experimental

Experiments were conducted at ambient temperature, unless otherwise noted. Solutions in organic solvents were dried with anhydrous magnesium sulfate. NMR spectra were obtained of solutions in deuteriochloroform (unless otherwise noted), using tetramethylsilane as chemical shift standard for ^1H (at 400 and 270 MHz) and ^{13}C (at 100 MHz). An external $CF^{35}Cl_3$ signal from fluorotrichloromethane was used for ^{19}F spectra (at 376 and 84.6 MHz). THF refers to dry tetrahydrofuran and brine refers to saturated aqueous sodium chloride. Solvents were evaporated under reduced pressure, except where noted. The stationary phase for chromatography was silica gel. A Kugelrohr apparatus was used for distillations and sublimations. Bps refer to the temperature of the Kugelrohr oven. Mps are uncorrected. A Corning 240 pH meter with a 3.7 mm, pH 0–14 Russell CLSCH11/1m electrode was used for the pH measurements. Compound **24** was prepared as described previously by us.[4]

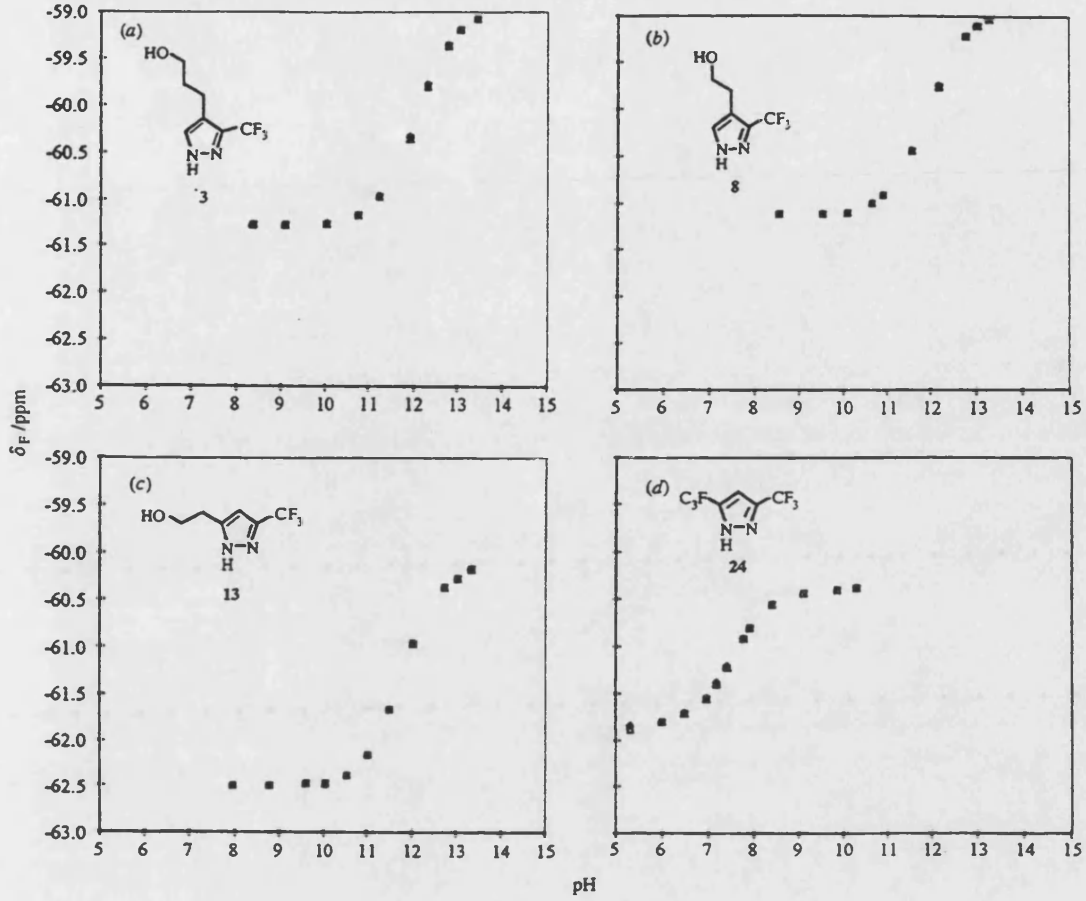


Fig. 1 δ_F–pH Relationships for the trifluoromethylpyrazoles (*a*) 3, (*b*) 8, (*c*) 13 and (*d*) 24

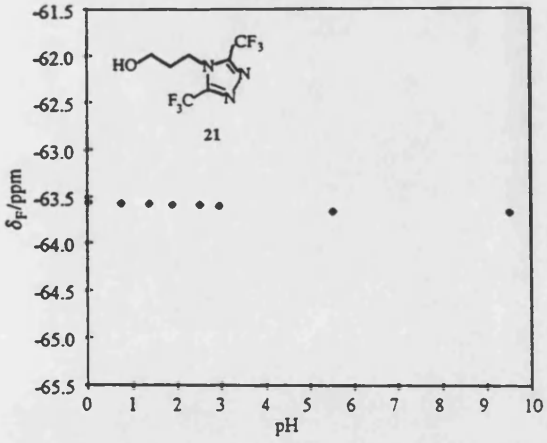


Fig. 2 δ_F–pH Relationship for the bis(trifluoromethyl)-4*H*-1,2,4-triazole 21

5-Trifluoroacetyl-3,4-dihydro-2*H*-pyran 2

Trifluoroacetic anhydride (17.9 g, 85 mmol) was added dropwise at 10 °C to 3,4-dihydro-2*H*-pyran 1 (5.00 g, 59 mmol) and pyridine (1.57 g, 20 mmol) in dichloromethane (40 cm^3) and the mixture was stirred for 18 h at ambient temperature. The mixture was washed twice with water and with saturated aqueous sodium hydrogen carbonate and was dried. Distillation gave 5-trifluoroacetyl-3,4-dihydro-2*H*-pyran 2 (10.84 g, 97%) as a colourless liquid: bp_{20} 95–100 °C (lit.,[8] bp_{10} 64.6 °C); $\delta_H(D_2O)$ 1.96 (2 H, br quintet, *J* 6 Hz, 3-H_2), 2.34 (2 H, t, *J* 6 Hz, 4-H_2), 4.21 (2 H, t, *J* 6 Hz, 2-H_2), 7.83 (1 H, s, 6-H); δ_F −70.37 (s); *m/z* (EI) 180 (M, 29%), 111 (M − CF_3, 100).

3-(3-Trifluoromethyl-1*H*-pyrazol-4-yl)propanol 3

The pyran 2 (1.0 g, 5.5 mmol) was heated under reflux with hydrazine hydrate (0.83 g, 16 mmol) in ethanol (3 cm^3) for 3 h. Distillation gave 3-(3-trifluoromethyl-1*H*-pyrazol-4-yl)propanol 3 (1.09 g, 99%) as a white solid: $bp_{0.4}$ 160 °C; mp 84–85 °C (lit.,[9] mp 85–87 °C); $\delta_H(D_2O)$ 1.79 (2 H, quintet, *J* 7 Hz, $CH_2CH_2CH_2$), 2.62 (2 H, t, *J* 7.3 Hz, pyrazole-CH_2), 3.59 (2 H, t, *J* 6.3 Hz, CH_2O), 7.66 (1 H, s, pyrazole 5-H); δ_F −61.31 (s) (HRMS: found M + H^+, 195.0745. $C_7H_{10}F_3N_2O$ requires *M* + H^+, 195.0745).

3-(3-Trifluoromethyl-1*H*-pyrazol-4-yl)propyl benzoate 4

Benzoyl chloride (540 mg, 3.9 mmol) was added dropwise over 15 min to the pyrazolylpropanol 3 (500 mg, 2.6 mmol) and triethylamine (5.2 g, 51.5 mmol) in dichloromethane (20 cm^3) at 0 °C and the mixture was allowed to warm to ambient temperature over 16 h. The mixture was washed with saturated aqueous sodium hydrogen carbonate, hydrochloric acid (2 M, five times) and brine and was dried. Chromatography (ethyl acetate–hexane, 1:1) of the evaporation residue gave the *ester* 4 (620 mg, 81%) as large white plates, mp 46–46.5 °C; δ_H 2.09 (2 H, quintet, *J* 7 Hz, $CH_2CH_2CH_2$), 2.80 (2 H, t, *J* 7.6 Hz, pyrazole-CH_2), 4.38 (2 H, t, *J* 6.1 Hz, CH_2O), 7.44 (2 H, br t, *J* 8 Hz, Ph 3,5-H_2), 7.54 (1 H, s, pyrazole 5-H), 7.56 (1 H, br t, *J* 8 Hz, Ph 4-H), 8.03 (2 H, br d, *J* 8 Hz, Ph 2,6-H_2); δ_F −61.30 (s); *m/z* (CI) 299 (M + H, 100%), 176 (M − $PhCO_2H$, 48), 105 (29) (HRMS: found M + H^+, 299.1007. $C_{14}H_{14}F_3N_2O_2$ requires *M* + H^+, 299.1007).

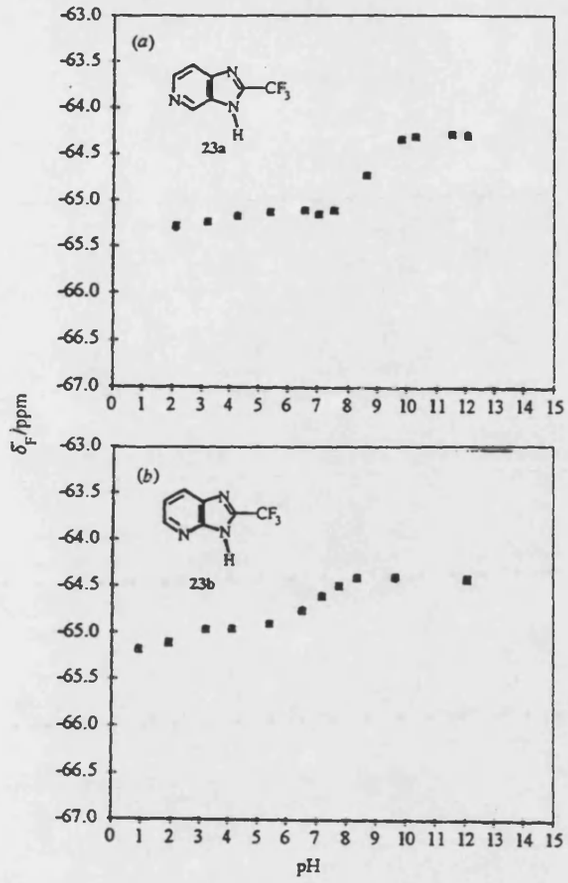


Fig. 3 δ_F–pH Relationships for 2-trifluoromethyl-3*H*-imidazol[4,5-*c*]-pyridines (*a*) **23a** and (*b*) **23b**

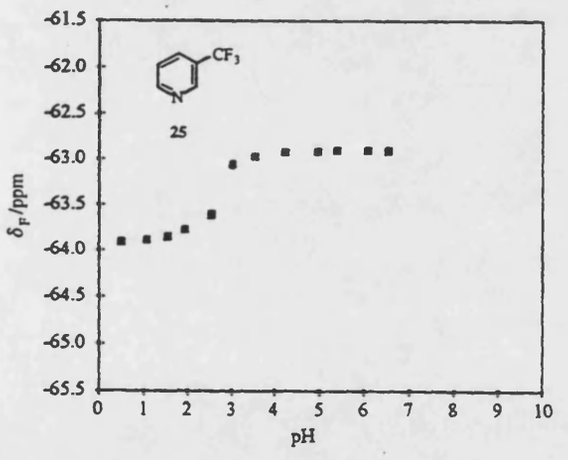


Fig. 4 δ_F–pH Relationship for 3-trifluoromethylpyridine **25**

3-Trifluoroacetyl-4,5-dihydrofuran 6

Trifluoroacetic anhydride (22.5 g, 107 mmol) was added dropwise with cooling to 2,3-dihydrofuran **5** (5.00 g, 71 mmol) and pyridine (1.88 g, 24 mmol) in dichloromethane (40 cm^3) and the mixture was stirred for 18 h. Distillation gave 3-trifluoroacetyl-4,5-dihydrofuran **6** (9.72 g, 82%) as a colourless liquid, bp_{20} 95–100 °C (lit.,[8] $bp_{10.5}$ 48.5 °C); δ_H 2.98 (2 H, tq, J_{H-H} 9.8, J_{H-F} 0.6 Hz, 4-H_2), 4.68 (2 H, t, J_{H-H} 9.8 Hz, 5-H_2), 7.64 (1 H, q, J_{H-F} 1.5 Hz, 2-H); δ_F −73.88 (br s); δ_C 26.93 (s, 4-C), 74.01 (s, 5-C), 114.37 (s, 3-C), 116.62 (q, J_{C-F} 190 Hz, CF_3), 163.84 (q, J_{C-F} 5 Hz, 2-C), 176.08 (q, J_{C-F} 36 Hz, C=O); *m/z* (EI) 166 (M + H, 45%), 97 (M − CF_3, 100) (HRMS: found M + H$^+$, 166.0239. $C_6H_5F_3O_2$ requires *M* + H$^+$, 166.0242).

2-{4,5-Dihydro-5-trifluoromethyl-5-[2-(3-trifluoromethyl-1*H*-pyrazol-4-yl)ethoxy]-1*H*-pyrazol-4-yl}ethanol 7

The furan **6** (1.0 g, 6.0 mmol) was heated under reflux with hydrazine hydrate (0.30 g, 6.0 mmol) in ethanol (13 cm^3) for 3 h. Evaporation of the solvent gave the *title compound* **7** (1.08 g, 99%) as a white solid, mp 84–85 °C; ν_{max}(Nujol)/cm^{-1} 3300, 3180, 1170; δ_H 1.82 (1 H, m) and 1.93 (1 H, m) (dihydropyrazole-CH_2), 2.79 (2 H, t, *J* 6.8 Hz, pyrazole-CH_2), 3.31 (1 H, br t, *J* 7 Hz, dihydropyrazole 4-H), 3.71 (2 H, t, *J* 6.8 Hz, pyrazole-CH_2CH_2O), 3.73 (2 H, m, dihydropyrazole-CH_2CH_2OH), 4.94 (3 H, 2 × NH + OH), 6.82 (1 H, s, dihydropyrazole 3-H), 7.67 (1 H, s, pyrazole 5-H); δ_F −82.57 (3 F, s, dihydropyrazole-CF_3), −62.20 (3 F, s, pyrazole-CF_3); δ_C 27.60 (s, CH_2), 28.36 (s, CH_2), 49.49 (s, dihydropyrazole 4-C), 60.92 (s, CH_2), 62.95 (s, CH_2), 92.93 (q, J_{C-F} 31 Hz, dihydropyrazole 5-C), 117.52 (s, pyrazole 4-C), 123.70 (q, J_{C-F} 268 Hz, CF_3), 125.68 (q, J_{C-F} 281 Hz, CF_3), 131.36 (s, CH), 140.95 (q, J_{C-F} 37 Hz, pyrazole 3-C), 146.30 (s, CH) (HRMS: found M + H$^+$, 361.1099. $C_{12}H_{15}F_6N_4O_2$ requires *M* + H$^+$, 361.1099).

2-(3-Trifluoromethyl-1*H*-pyrazol-4-yl)ethanol 8

Compound **7** (1.00 g, 2.8 mmol) was heated under reflux with hydrochloric acid (9 M, 0.05 cm^3) in ethanol (12 cm^3) for 16 h. The solvent and reagent were evaporated to give 2-(3-*trifluoromethyl*-1H-*pyrazol*-4-*yl*)*ethanol* **8** (990 mg, 99%) as a pale yellow solid, mp 90–91 °C; δ_H[$(CD_3)_2SO$] 2.65 (2 H, br t, *J* 7 Hz, pyrazole-CH_2), 3.54 (2 H, t, *J* 7 Hz, CH_2O), 4.9 (1 H, br, OH), 7.78 (1 H, br s, pyrazole 5-H); δ_F[$(CD_3)_2SO$] −59.66 (s); *m/z* (EI) 180 (M, 30%), 159 (M − CH_2OH, 100) (HRMS: found M, 180.0515. $C_6H_7F_3NO$ requires *M*, 180.0510).

6-*tert*-Butyldiphenylsilyloxy-1,1,1-trifluorohex-3-yn-2-one 11a

Butyllithium (2.5 M in hexanes; 13 cm^3, 32.4 mmol) was added to 4-*tert*-butyldiphenylsilyloxybut-1-yne **10a**[10] (10.0 g, 32.4 mmol) in THF (50 cm^3) at −78 °C and the mixture was stirred at this temperature for 30 min. 2,2,2-Trifluoroethyl trifluoroacetate (7.00 g, 35.7 mmol) in THF (60 cm^3) was added, followed immediately by boron trifluoride–diethyl ether (5.65 g, 40 mmol), and the mixture was stirred at −78 °C for 90 min. Saturated aqueous ammonium chloride (18 cm^3) was added and the mixture was allowed to warm to ambient temperature. The THF was evaporated and the residue, in diethyl ether, was washed with water and twice with brine and dried. Evaporation of the solvent gave the *ketone* **11a** (12.43 g, 95%) as a colourless viscous oil; δ_H 1.06 (9 H, s, Bu^t), 2.72 (2 H, t, *J* 6.3 Hz, $CH_2C{\equiv}C$), 3.85 (2 H, t, *J* 6.3 Hz, CH_2O), 7.3–7.7 (10 H, 2 × Ph-H_5); δ_F −78.63 (s) (HRMS: found M + H$^+$, 405.1498. $C_{22}H_{24}F_3O_2Si$ requires *M* + H$^+$, 405.1498).

6-Benzyloxy-1,1,1-trifluorohex-3-yn-2-one 11b

Butyllithium (2.5 M in hexanes; 18 cm^3, 45 mmol) was added to 4-benzyloxybut-1-yne **10b**[11] (7.2 g, 45 mmol) in THF (100 cm^3) at −78 °C and the mixture was stirred at this temperature for 30 min. 2,2,2-Trifluoroethyl trifluoroacetate (9.7 g, 50 mmol) in THF (70 cm^3) was added, followed immediately by boron trifluoride–diethyl ether (8.5 g, 60 mmol) and the mixture was stirred at −78 °C for 90 min. Saturated aqueous ammonium chloride (30 cm^3) was added and the mixture was allowed to warm to ambient temperature. The THF was evaporated and the residue, in dichloromethane, was washed with water and twice with brine and dried. Evaporation of the solvent gave the *ketone* **11b** (11.13 g, 97%) as a pale yellow oil; δ_H 2.80 (2 H, t, *J* 6.6 Hz, $CH_2C{\equiv}C$), 3.69 (2 H, t, *J* 6.6 Hz, OCH_2CH_2), 4.58 (2 H, s, $PhCH_2O$), 7.30–7.35 (5 H, m, Ph-H_5); δ_F −78.65 (s) (HRMS: found M − H, 255.0633. $C_{13}H_{10}F_3O_2$ requires *M* − H, 255.0633).

5-(2-*tert*-Butyldiphenylsilyloxyethyl)-3-trifluoromethyl-1*H*-pyrazole 12a

The ketone **11a** (378 mg, 0.93 mmol) was heated under reflux with hydrazine hydrate (73 mg, 0.93 mmol) in ethanol (2.2 cm^3) for 90 min. The evaporation residue, in dichloromethane, was dried and the solvent was evaporated to give the *pyrazole* **12a** (385 mg, 99%) as a colourless oil; δ_H 1.06 (9 H, s, Bu'), 2.88 (2 H, t, *J* 5.7 Hz, pyrazole-CH_2), 3.89 (2 H, t, *J* 5.7 Hz, CH_2O), 6.33 (1 H, s, pyrazole 4-H), 7.3–7.7 (10 H, m, 2 × Ph-H_5); δ_F −62.31 (s); δ_C 19.01 (s, CMe_3), 26.74 (s, 3 × CH_3), 28.44 (s, pyrazole-CH_2), 62.53 (s, CH_2O), 102.69 (s, pyrazole 4-C), 121.44 (q, J_{C-F} 268 Hz, CF_3), 127.80 (s, 2 × Ph 3,5-C_2), 129.89 (s, 2 × Ph 4-C), 132.83 (s, 2 × Ph 1-C), 135.41 (s, 2 × Ph 2,6-C_2), 142.87 (q, *J* 37 Hz, pyrazole 3-C), 143.28 (s, pyrazole 5-C) (HRMS: found M + H^+, 419.1767. $C_{22}H_{26}F_3N_2OSi$ requires *M* + H^+, 419.1767).

5-(2-Benzyloxyethyl)-3-trifluoromethyl-1*H*-pyrazole 12b

The ketone **11b** (500 mg, 1.95 mmol) was boiled under reflux with hydrazine hydrate (153 mg, 1.95 mmol) in ethanol (4.6 cm^3) for 1 h. The evaporation residue, in dichloromethane, was dried and the solvent was evaporated to give the *pyrazole* **12b** (480 mg, 91%) as a pale yellow oil; δ_H 2.91 (2 H, t, *J* 5.9 Hz, pyrazole-CH_2), 3.68 (2 H, t, *J* 5.9 Hz, OCH_2CH_2), 4.51 (2 H, s, $PhCH_2O$), 6.33 (1 H, s, pyrazole 4-H), 7.2–7.4 (5 H, m, Ph-H_5); δ_F −62.35 (s); δ_C 25.96 (s, pyrazole-CH_2), 68.31 (s, OCH_2), 73.21 (s, OCH_2), 102.33 (s, pyrazole 4-C), 121.46 (q, J_{C-F} 268 Hz, CF_3), 127.75 (s, Ph 2,6-C_2), 127.91 (s, Ph 4-C), 128.48 (s, Ph 3,5-C_2), 137.44 (s, Ph 1-C), 142.66 (q, J_{C-F} 39 Hz, pyrazole 3-C), 143.02 (s, pyrazole 5-C) (HRMS: found M + H^+, 271.1058. $C_{13}H_{14}F_3N_2O$ requires *M* + H^+, 271.1058).

2-(3-Trifluoromethyl-1*H*-pyrazol-5-yl)ethanol 13

The benzyl protected compound **12b** (320 mg, 1.2 mmol) and perchloric acid (60% in water, 0.01 cm^3) in methanol (4.0 cm^3) and THF (1.0 cm^3) were treated with hydrogen in the presence of palladium on charcoal (10%, 100 mg) for 70 min. The mixture was filtered through Celite and the solvent was evaporated from the combined filtrate and methanol washings. Recrystallisation of the residue from chloroform–ethanol gave 2-(3-*trifluoromethyl*-1H-*pyrazol*-5-*yl*)*ethanol* **13** (200 mg, 94%) as a white solid; mp 85–86 °C; δ_H[$(CD_3)_2CO$] 2.98 (2 H, dt, *J* 0.5, 5.7 Hz, pyrazole-CH_2), 4.12 (2 H, t, *J* 5.7 Hz, CH_2O), 6.40 (1 H, br s, pyrazole 4-H); δ_F −61.83 (br s) (HRMS: found M, 180.0541. $C_6H_7F_3N_2O$ requires *M*, 180.0510. Found M − CH_2O, 150.0436. $C_5H_5F_3N_2$ requires *M* − CH_2O, 150.0404).

3-Benzyloxypropyl toluene-*p*-sulfonate 15

3-Benzyloxypropanol **14** (7.61 g, 46 mmol) was stirred with powdered potassium hydroxide (2.60 g, 46 mmol) and toluene-*p*-sulfonyl chloride (8.73 g, 46 mmol) in dry diethyl ether (50 cm^3) for 6 h. Powdered potassium hydroxide (2.0 g, 36 mmol) was added and stirring continued for a further 2.5 h. The mixture was filtered and the filtrate was washed twice with water and dried. The solvent was evaporated to give 3-*benzyloxypropyl toluene*-p-*sulfonate* **15** (13.3 g, 91%) as white crystals, mp < 25 °C; δ_H 1.88 (2 H, quintet, *J* 6.1 Hz, $CH_2CH_2CH_2$), 2.45 (3 H, s, CH_3), 3.52 (2 H, t, *J* 6.0 Hz, $BnOCH_2$), 4.19 (2 H, t, *J* 6.2 Hz, $TsOCH_2$), 4.43 (2 H, s, $PhCH_2O$), 7.25–7.35 (7 H, m, Ph-H_5 + Tol 3,5-H_2), 7.82 (2 H, d, *J* 8.3 Hz, Tol 2,6-H_2); *m/z* (CI) 321 (M + H, 17%), 181 (9), 155 (10), 91 (100).

***N*-(3-Benzyloxypropyl)-2,2,2-trifluoroacetamide 16**

Potassium *tert*-butoxide (770 mg, 6.9 mmol) was added to trifluoroacetamide (780 mg, 6.9 mmol) in THF (20 cm^3), followed by the tosylate **15** (2.0 g, 6.2 mmol) and sodium iodide (50 mg). The solution was stirred for 4 h and was then cooled to 0 °C. Diethyl ether (20 cm^3) and water (10 cm^3) were added and the mixture was acidified to pH 1 by addition of hydrochloric acid, the layers were separated and the organic phase was washed twice with water and once with brine. The solution was dried and the solvent was evaporated to give the *amide* **16** (1.67 g, 97%) as a pale yellow oil; ν_{max}(liquid film)/cm^{-1} 3350, 1715, 1170; δ_H 1.87 (2 H, m, $CH_2CH_2CH_2$), 3.50 (2 H, m, CH_2N), 3.66 (2 H, t, *J* 5.4 Hz, $BnOCH_2$), 4.45 (2 H, s, $PhCH_2O$), 7.34 (5 H, m, Ph-H_5); δ_F −76.71 (s). This material was used immediately without further characterisation.

3-Benzyloxypropylamine 17

The amide **16** (1.60 g, 5.8 mmol) was heated under reflux with sodium hydroxide (1.00 g, 50 mmol) in methanol (40 cm^3) for 4 h. The solvent was evaporated. The residue, in diethyl ether, was washed with saturated aqueous sodium hydrogen carbonate and dried. The solvent was evaporated and the residue was distilled to give the primary amine **17** (630 mg, 66%) as a colourless liquid, $bp_{0.6}$ 85–90 °C (lit.,[19] $bp_{0.75}$ 93.5 °C); δ_H 1.43 (2 H, br, NH_2), 1.75 (2 H, quintet, *J* 6.2 Hz, $CH_2CH_2CH_2$), 2.82 (2 H, t, *J* 6.8 Hz, CH_2N), 3.55 (2 H, t, *J* 6.2 Hz, $BnOCH_2$), 4.51 (2 H, s, $PhCH_2O$), 7.33 (5 H, m, Ph-H_5).

2,5-Bis(trifluoromethyl)-1,3,4-oxadiazole 19

1,2-Bis(trifluoroacetyl)hydrazine **18** (6.94 g, 31 mmol) was mixed intimately with phosphorus pentoxide (15 g) and the mixture was covered with a further layer of phosphorus pentoxide (10 g). The mixture was heated to 300 °C in a distillation apparatus and the volatile materials were condensed in a trap at −78 °C. The distillate was redistilled (Kugelrohr) from calcium hydride to give the oxadiazole **18** (4.01 g, 74%) as a colourless liquid, bp 65 °C (lit.,[14] bp 65 °C); δ_F −65.34 (s).

3,5-Bis(trifluoromethyl)-4-(3-benzyloxypropyl)-4*H*-1,2,4-triazole 20

The oxadiazole **19** (371 mg, 1.8 mmol) was added dropwise to 3-benzyloxypropylamine **17** (300 mg, 1.8 mmol) in methanol (0.6 cm^3) at 0 °C over 5 min. The mixture was heated under reflux for 9 d. Distillation gave the *triazole* **20** (270 mg, 42%) as a colourless liquid, $bp_{0.6}$ 150–155 °C; δ_H 2.12 (2 H, m, $CH_2CH_2CH_2$), 3.57 (2 H, t, *J* 5.5 Hz, $BnOCH_2$), 4.38 (2 H, t, *J* 8.2 Hz, NCH_2), 4.51 (2 H, s, $PhCH_2O$), 7.30 (5 H, m, Ph-H_5); δ_F −62.80 (s) (HRMS: found M + H^+, 354.1041. $C_{14}H_{14}F_6N_3O$ requires *M* + H^+, 354.1041).

3-[3,5-Bis(trifluoromethyl)-4*H*-1,2,4-triazol-4-yl]propanol 21

The benzyloxypropyltriazole **20** (200 mg, 570 μmol) in ethanol (2.5 cm^3) and perchloric acid (60% in water, 0.01 cm^3) were stirred under an atmosphere of hydrogen with palladium on charcoal (10%, 30 mg) for 29 h. The catalyst was removed by filtration through Celite. Sodium hydrogen carbonate (1.0 g) was added to the combined filtrate and ethanol washings and the mixture was stirred for 1 h. The mixture was filtered and the solvent was evaporated from the filtrate to give the *alcohol* **21** (140 mg, 94%) as a white solid, mp < 25 °C; $\delta_H(D_2O)$ 2.14 (2 H, m, $CH_2CH_2CH_2$), 3.74 (2 H, t, *J* 5.8 Hz, CH_2O), 4.48 (2 H, t, *J* 7.9 Hz, NCH_2); δ_F −63.49 (s) (HRMS: found M + H^+, 264.0720. $C_7H_8F_6N_3O$ requires *M* + H^+, 264.0720).

2-Trifluoromethyl-3*H*-imidazo[4,5-*c*]pyridine 23a

3,4-Diaminopyridine **22a** (1.00 g, 9.2 mmol) was heated under reflux in trifluoroacetic acid (10 cm^3) for 2 d. Evaporation of excess reagent and sublimation (200 °C, 0.1 Torr) gave 2-trifluoromethyl-3*H*-imidazo[4,5-*c*]pyridine **23a** (1.71 g, 98%) as a white solid, mp 159–161 °C (lit.,[20] mp 290–291 °C) (Found: C, 44.70; H, 2.12; N, 22.10. $C_7H_4F_3N_3$ requires C, 44.93; H, 2.15; N, 22.46%); δ_H[$(CD_3)_2SO$] 7.98 (1 H, d, *J* 6.6 Hz, 7-H), 8.27 (1 H, d, *J* 6.6 Hz, 6-H), 9.25 (1 H, s, 4-H), 14.2 (1 H, br, NH); δ_C[$(CD_3)_2SO$] 114.32 (s, 7-C), 120.91 (q, J_{C-F} 270 Hz, CF_3), 129.42 (m, 2-C), 133.35 (s, 6-C), 142.15 (s, 4-C), 154.02 (br, C_q) (the signal for one quaternary carbon was not observed); δ_F[$(CD_3)_2SO$] −63.60 (s) (HRMS: found M, 187.0364. $C_7H_4F_3N_3$ requires *M*, 187.0357).

2-Trifluoromethyl-3*H*-imidazo[4,5-*b*]pyridine 23b

2,3-Diaminopyridine **22b** (1.00 g, 9.2 mmol) was heated under reflux in trifluoroacetic acid (10 cm^3) for 2 d. Evaporation of excess reagent and sublimation (200 °C, 0.1 Torr) gave 2-*trifluoromethyl*-3H-*imidazo*[4,5-b]*pyridine* **23b** (1.67 g, 97%) as a yellow solid, mp 240–241 °C; δ_H[$(CD_3)_2SO$] 7.48 (1 H, dd, *J* 8.4, 4.8 Hz, 6-H), 8.29 (1 H, br d, *J* 8.1 Hz, 7-H), 8.59 (1 H, dd, *J* 4.8, 1.5 Hz, 5-H), 14.6 (1 H, br, NH); δ_C[$(CD_3)_2SO$] 119.07 (q, J_{C-F} 271 Hz, CF_3), 119.59 (br, 6,7-C_2), 142.73 (q, J_{C-F} 44 Hz, 2-C), 145.76 (s, 5-C) (signals due to the quaternary carbons at 3a and 7a were not observed); δ_F[$(CD_3)_2SO$] −63.65 (s) (HRMS: found M, 187.0353. $C_7H_4F_3N_3$ requires *M*, 187.0357).

5-Fluoro-2-trifluoromethyl-1*H*-benzimidazole 23c

4-Fluorobenzene-1,2-diamine **22c** (1.00 g, 7.9 mmol) was heated under reflux in trifluoroacetic acid (10 cm^3) for 5 d. Evaporation of excess reagent and sublimation (200 °C, 12 Torr) yielded 5-fluoro-2-trifluoromethyl-1*H*-benzimidazole **23c** (1.56 g, 95%) as a white solid, mp 174–175 °C (lit.,[21] mp 219–220 °C) (Found: C, 46.80; H, 1.93; N, 13.50. $C_8H_4F_4N_2$ requires C, 47.07; H, 1.98; N, 13.72%); δ_H[$(CD_3)_2SO$] 7.28 (1 H, dt, *J* 9.2, 2.1 Hz, 6-H), 7.56 (1 H, br d, *J* 8.9 Hz, 7-H), 7.79 (1 H, m, 4-H), 14.2 (1 H, br, NH); δ_F[$(CD_3)_2SO$] −63.20 (s, CF_3), −116.79 (br m, 5-F) (HRMS: found M^+, 204.0321. $C_8H_4F_4N_2$ requires *M*, 204.0311).

2,5-Bis(trifluoromethyl)-1*H*-benzimidazole 23d

4-(Trifluoromethyl)benzene-1,2-diamine **22d** (1.00 g, 5.68 mmol) was heated under reflux in trifluoroacetic acid (10 cm^3) for 5 d. Evaporation of excess reagent and sublimation (150 °C, 10 Torr) afforded 2,5-bis(trifluoromethyl)-1*H*-benzimidazole **23d** (1.24 g, 89%) as a white solid, mp 159–161 °C (lit.,[22] 198–199 °C) (Found: C, 42.60; H, 1.55; N, 11.10. $C_9H_4F_6N_2$ requires C, 42.54; H, 1.59; N, 11.02%); δ_H[$(CD_3)_2SO$] 7.72 (1 H, dd, *J* 8.8, 1.8 Hz, 6-H), 7.95 (1 H, d, *J* 8.4 Hz, 7-H), 8.16 (1 H, br s, 4-H), 14.3 (1 H, br, NH); δ_F[$(CD_3)_2SO$] −63.44 (s, 2-CF_3), −59.72 (br s, 5-CF_3) (HRMS: found M, 254.0254. $C_9H_4F_6N_2$ requires *M*, 254.0279).

Studies on the effect of pH on ^{19}F NMR chemical shift

The heterocycles (*ca.* 2 mg) were dissolved in aqueous buffers (*ca.* 1 cm^3) of various compositions and pHs. The ^{19}F NMR spectra of samples (0.6 cm^3) were acquired using a JEOL EX400 spectrometer. An external reference (the $CF^{35}Cl_3$ signal in fluorotrichloromethane in deuteriochloroform) was used before and after each determination but no drift was noted. The chemical shifts were found to be independent of the composition of the inorganic buffers and independent of the concentrations of the buffers and trifluoromethylheterocycle in the range 100 to 400 mM for the buffer and 5 to 20 mM for the trifluoromethyl heterocycles. The effect of temperature was insignificant in the range 25 to 37 °C, so spectra were recorded routinely at 25 °C. Each determination of δ_F was performed in triplicate for each pH value. The pHs of the solutions containing the test compound, the buffer and 5% D_2O were measured immediately before the NMR experiments, using a pH meter with an electrode suitable for small volumes. 132K Data points were used in each NMR data acquisition to ensure high digital resolution (*ca.* 0.4 Hz per data point).

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Modulation of the Cytotoxic Activity of Murine Macrophages by Flavones

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MODULATION OF THE CYTOTOXIC ACTIVITY OF MURINE MACROPHAGES BY FLAVONES

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The effect of flavonoids (13 flavones and 2 isoflavones) on the production of nitrite and on luminol - dependent chemiluminescence by murine activated peritoneal macrophages (F4/80 positive) was studied *in vitro*. The accumulated nitrite as a stable final product of nitric oxide (NO) was determined by the Griess reaction. Nitrite production was inhibited by flavonoids. 3'-Amino-4'-hydroxyflavone and 3-hydroxyflavone were the most potent inhibitors of nitrite production. These compounds also inhibited chemiluminescence. Chemiluminescence was used in this study as an indicator for production of reactive oxygen species (ROIs) by macrophages. These data suggested a causative connection between NO and ROIs production in macrophages. Also these results show that the flavones can modulate the immune responses and the inflammatory reactions by controlling production of NO.

INTRODUCTION

The production of reactive oxygen (ROIs) and reactive oxynitrogen (RONIs) intermediates by macrophages is critical to host defence. Upon stimulation with both soluble and particulate matter, oxidative metabolism is stimulated in the macrophage resulting in the respiratory burst which is accompanied by activation of an NADPH-oxidising enzyme. This enzyme catalyses the reduction of molecular oxygen to superoxide anion (O_2^-) and the burst is paralleled by consumption of oxygen. O_2^- is the precursor of other ROIs, including hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^{\cdot}$). Oxidation of cellular molecules by ROIs contributes to killing of phagocytized microbes and can cause severe toxicity to cancer cells (Fridovich, 1986). Until 1987, the only inducible biochemical mechanism that could explain activated macrophage cytotoxicity was the synthesis of ROIs by NADPH oxidase. Subsequently, the production of nitric oxide ($^{\cdot}NO$) from L-arginine by an immune/ inflammatory isoform nitric oxide synthase (iNOS) was described by Hibbs, Vavrin and Taintor (1987a, 1987b). Biosynthesis of NO may lead to the production of other RONIs, including nitrosonium (NO^+) and nitroxyl (NO^-) ions, nitrogen dioxide (NO_2), peroxynitrite ($ONOO^-$), and S-nitrosothiols (Stamler, Singel & Loscalzo, 1992). RONIs kill some cancer cells and are the ultimate effector molecules in the host defence against many intracellular pathogens, such as *Toxoplasma gondii*, *Leishmania*, and *Mycobacterium tuberculosis* and extracellular pathogens, such as *Cryptococcus neoformans* and *Schistosoma mansoni*.

However, both ROIs and RONIs are potentially toxic to the host making regulation of the production of these highly reactive molecules of utmost importance to host survival. NO, O_2^- and its intermediates produced by activated phagocytes may play an important role in the multistage carcinogenesis process, triggered by chronic infection and inflammation. Inoue and Kawanishki (1995) suggest that NO reacts with O_2^- to form $ONOO^-$ and the $ONOO^-$ induces oxidative DNA damage through an active intermediate of the reactivity which is similar to $HO^{\cdot}$. Our earlier studies have shown that functions of activated macrophages can be regulated by flavones (2-phenyl-4*H*-1-benzopyran-4-ones) (Middleton & Kandaswami, 1992). Flavones are a group of naturally occurring compounds, widely distributed as secondary metabolites in the plant kingdom. They have been ac-

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knowledged as having interesting medicinal properties, such as antiinflammatory, antiallergic, antiviral, antibacterial, and antitumour activities (Havesteen, 1983). Flavones have also been the scavengers of free radicals and act as natural antioxidants. They have also been shown to inhibit oxido-reductase, thus preventing the formation of free radicals resulting from the reduction of oxygen. These two mechanisms can account for the role of many flavones in protecting cells from oxidative damage (Król, Czuba & Scheller, 1986).

The present study was aimed to investigation of the role of flavones in the regulation of RONIs and RONIs release from activated murine macrophages. Chemiluminescence was used in this study as an indicator for the productions of ROIs by macrophages stimulated by PMA as stimulant for oxygen metabolism through the activation of protein kinase C (PKC). RONIs release from activated macrophages was determined by nitrite as a stable end product of 'NO.

MATERIAL AND METHODS

Mice

Specific pathogen-free 8- to 10-week old BALB/c male mice were purchased from the Institute of Oncology, Gliwice, Poland.

Reagents

Flavone, 4'-aminoflavone, 3'-amino-4'-hydroxyflavone, and 3'-amino-4'-methoxyflavone were synthesised as previously described (Cunningham, Threadgill, Groundwater, Dale & Hickman, 1992; Król, Czuba, Threadgill, Cunningham & Shani, 1995a). Flavone-8-acetic acid (FAA) was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, USA, through the courtesy of Dr. K. Paull. 4'-amino-6-hydroxyflavone, daidzein, genistein, lipopolysaccharide (LPS) from *E. coli* (serotype 0127 : B8), Concanavalin A (Con A), N^G-monomethyl-L-arginine monoacetate salt (N^GMMA), superoxide dismutase (SOD) from human placental (4750 U/mg) were purchased from Calbiochem (La Jolla, CA, USA). 3-Hydroxyflavone and other flavones were purchased from Roth Chem. (Karlsruhe, Germany). Hanks' balanced salt solution (HBSS) without phenol red, phosphate-buffered saline solution (PBS), RPMI 1640 medium without phenol red, and heat-inactivated fetal calf serum (FCS) low in endotoxin, penicillin – streptomycin (10 000 IU/ml – 10 000 µg/ml) were purchased from GIBCO BRL Life Technologies Ltd. (Paisley, UK). Neutral red solution (0.5% aqueous solution of the sodium salt), N-(1-naphthyl)-ethylenediamine dihydrochloride, sulphanilamide and heparin sodium (pyrogen free) were purchased from Serva Chemicals (Heidelberg, Germany). Phorbol myristate acetate (PMA), kit for α-naphthyl acetate esterase determination and dimethylsulphoxide (DMSO) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) was bought from LKB (Turku, Finland). The monoclonal antibody (mAb) anti-mouse Thy-1,2 fluorescein conjugate was purchased from Becton Dickinson Systems (Mountain View, CA, USA). The mAb F4/80 anti-mouse macrophages fluorescein conjugate was purchased from SEROTEC Ltd. (Oxford, UK). Fluid thioglycolate medium was purchased from DIFCO Lab. (Detroit, MI, USA).

Collection and cultivation of mouse peritoneal exudate macrophages

Macrophages were obtained from mice given an i.p. injection of sterile thioglycolate broth (1 ml) 4d prior to harvest and the solution of Con A (100 µg/ml) in PBS (1 ml) 18 h prior to harvest. Mice were sacrificed by cervical dislocation, and cells were collected by washing the peritoneum with PBS (5ml). The macrophages populations were enriched by adherence to plastic or glass 24-well plates (Falcon Becton Dickinson, Lincoln Park, NJ, USA) (nitrite assay), 16-well TC chamber slides (Nunc, Roskilde, Denmark) (morphological study of macrophages) or test tubes (chemiluminescence) with calculated 10^6 macrophages per well or tube. Non-adherent cells were removed after 2 h of incubation in RPMI 1640 without phenol red, supplemented with 10% FCS, penicillin (100 U/ml) and streptomycin (100 µg/ml) (culture medium) at 37°C in a humidified atmosphere of 5% CO_2 in air. The adherent population cells were assessed by May-Grünwald/Giemsa staining and biochemical criteria whereas immunological specificity (mAbs to mouse leukocyte differentiation antigens) by fluorescence microscopy.

Nitrite assay

The accumulated nitrite as a stable end product of NO was determined colorimetrically by a diazotization reaction using the standard Griess reagent (Green, Wagner, Głogowski, Skipper, Wishnak & Tannenbaum, 1982), as described in detail previously (Król, Czuba, Threadgill, Cunningham & Pietsz, 1995a) after 48 h culture of macrophages

in the absence or presence of various flavonoids, SOD, N^GMMA in the presence of LPS. The absorbance of culture medium and Griess reagent at 550 nm was determined with automated microplate reader ELx 800 (Bio-Tek Instruments Inc., Winooski, VT, USA). Nitrite content was determined by using sodium nitrite as standard. Data were expressed as nmol nitrite per 10^6 cells originally plated. In all experiments, the nitrite content in wells containing medium without cells was measured and subtracted.

Chemiluminescence

Chemiluminescence was measured as described previously (Król, Czuba, Scheller, Gabryś, Grabiec & Shani, 1990; Czuba, Król, Scheller & Shani, 1992) using a special low noise-count rate photomultiplier 9514s (EMI, Middlesex, UK). The sample volume was usually 1 ml. The reactions were initiated by dispensing aliquots of solutions of luminol (100 μM) to macrophages (10^6) and tested compounds in HBSS in glass test tubes. The resting intensity of chemiluminescence was recorded after 5 min. PMA solution was then added to give a final concentration of 0.8 μM. The light emitted was then recorded continuously for 20 min.

RESULTS

The adherent cells populations contained > 98% macrophages as assessed by May-Grünwald/Giemsa staining and biochemical criteria (nonspecific esterase staining), > 98% F4/80-positive, and < 2% T-lymphocytes by fluorescence microscopy after fluorescent antibody labelling with anti Thy-1,2 mAb. More than 98% of the cells were viable as determined by exclusion of neutral red, also after incubation with all reagents.

Con A-stimulated macrophages produced L-arginine-dependent NO in the presence of LPS (100 ng/ml). The production of nitrite as a stable final product of NO was 44.2 ± 1.0 nmol/10^6 cells after 48 h. N^GMMA (300 μM) inhibited nitrite production by 87 ± 2% (Table 1) SOD (200 U) also decreased the production of nitrite. In an other experiment, upon addition of PMA (0.8 μM), macrophages luminol-dependent chemiluminescence increased 25 times over baseline light emission, reaching the maximum about 9 min after addition of the stimuli. The NOS inhibitor, N^GMMA (300 μM) plus SOD, significantly diminished route and peak intensities of PMA-stimulated macrophages chemiluminescence (85 ± 1%) (Table 1).

In the next experiment, the flavonoids were investigated for their involvement in release of NO from Con A and LPS-activated macrophages. The structures of the flavonoids used in this work are shown in Table 2. Concentrations of the flavonoids in the range 10–50 μM reduced the production of nitrite (Table 3). 3-Hydroxyflavone and 3'-amino-4'-hydroxyflavone were the most potent inhibitors. Genistein, an isoflavone that inhibits tyrosine-specific protein kinases, and daidzein, an isoflavone analogue that is inactive against these tyrosine kinases, also significantly decreased the production of nitrite.

Hence, inhibition of production of nitrite is unlikely to be due to toxicity of the compounds. Additionally, the flavones and isoflavones had no quenching effect on the Griess reagent at the concentration used.

The purpose of the present study was also to evaluate quantitatively the activity of flavonoids on the chemiluminescent activity of PMA-stimulated macrophages.

Table 1

Inhibitory effects of the superoxide dismutase and of N^G-monomethyl-L-arginine on the nitrite and luminol-dependent chemiluminescence production by murine macrophages

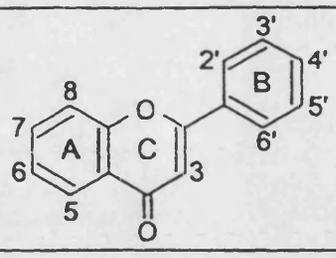
Inhibitor	Inhibition (%)	
	NO_2^- production[a]	CL production[b]
SOD (200 U)	10 ± 1	81 ± 1
N^GMMA (300 μM)	87 ± 2	45 ± 1
SOD (200 U) + N^GMMA (300 μM)	88 ± 2	85 ± 1

[a] Nitrite concentrations in the culture medium were measured after 48 h. Macrophages were incubated with LPS (100 ng/ml) in the presence of the inhibitors. Concentration of nitrite (nmol/10^6 cells, mean ± SD of three wells from three independent experiments) produced by control macrophages were 44.2 ± 1.0.

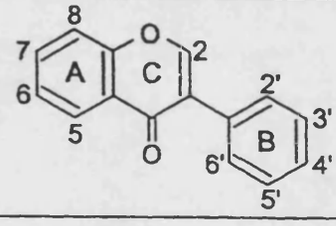
[b] The intensity of CL was determined by measuring counts/min and by calculating the area under CL intensity curve (integral counts). The rates of CL produced by 1×10^6 macrophages for 20 min is the absence of the inhibitors were 85000 ± 2000 (integral counts). Results are expressed as mean ± SD, n = 2–4.

Table 2

Structures of flavonoids evaluated.



flavo-noid	name	substituent						
		3	5	6	7	8	3'	4'
1	Flavone	H	H	H	H	H	H	H
2	3-Hydroxyflavone	OH	H	H	H	H	H	H
3	Flavone-8-acetic acid	H	H	H	H	CH_3CO_2H	H	H
4	4'-Aminoflavone	H	H	H	H	H	H	NH_2
5	3'-Amino-4'-hydroxyflavone	H	H	H	H	H	NH_2	OH
6	3'-Amino-4'-metoxyflavone	H	H	H	H	H	NH_2	OCH_3
7	4'-Amino-6-hydroxyflavone	H	H	OH	H	H	H	NH_2
8	Chrysin	H	OH	H	OH	H	H	H
9	Galangin	OH	OH	H	OH	H	H	H
10	Genkwanin	H	OH	H	OCH_3	H	H	OH
11	Kaempferol	OH	OH	H	OH	H	H	OH
12	Kaempferide	OH	OH	H	OH	H	H	OCH_3
13	Quercetin	OH	OH	H	OH	H	OH	OH



		substituent		
		5	7	4'
14	Daidzein	H	OH	OH
15	Genistein	OH	OH	OH

The level of inhibition of all fourteen compounds tested is presented in Table 4. 3-Hydroxyflavones (3-hydroxyflavone, galangin, kaempferol, kaempferide and quercetin) and 3'-amino-4'-hydroxyflavone were the most potent inhibitors. PMA-stimulated production of chemiluminescence was enhanced to a small but statistically insignificant extent in the presence of low (10 μM) concentrations of 4'-aminoflavone and 3'-amino-4'-methoxyflavone. However, PMA-stimulated production of chemiluminescence was progressively inhibited in the presence of increasing concentrations of these agents.

DISCUSSION

The toxicity of NO is primarily determined in the presence of H_2O_2 rather than O_2^-. This synergism may have important implications for the understanding of the pathophysiology of sepsis, reperfusion injury and shock. Locally disregulated productions of either species (e.g. after the exposure of cytokines) may be fatal when are produced together, but regulate normal cellular functions when acting separatly. A second possible explanation may arise from a regulated coproduction when cytotoxicity is required. Macrophages are capable

Table 3

Effects of flavonoids on the production of nitrite by murine macrophages

Flavonoid	Inhibition (%)[a]		
	Concentration (μM)		
	10	20	50
1	2 ± 1	3 ± 1	75 ± 10
2	ND	96 ± 1	ND
3	−1 ± 1	3 ± 2	9 ± 4
4	49 ± 1	66 ± 1	86 ± 3
5	30 ± 4	89 ± 3	ND
6	3 ± 1	23 ± 1	83 ± 7
7	3 ± 1	ND	86 ± 2
8	3 ± 1	86 ± 1	80 ± 3
9	15 ± 2	27 ± 3	ND
10	42 ± 3	67 ± 8	ND
11	22 ± 5	70 ± 2	ND
12	22 ± 6	80 ± 6	ND
13	4 ± 1	2 ± 1	33 ± 20
14	ND	ND	97 ± 2
15	ND	ND	57 ± 9

[a]Macrophages were incubated with LPS (100 ng/ml), without flavonoids or with flavonoids, for 48 h. Concentration of nitrite (nmol 10^6 cells, mean ± SD of three wells from three independent experiments) produced by control macrophages were 42.8 ± 5.9.
ND: not determined.

Table 4

Effects of flavonoids on luminol – dependent chemiluminescence of murine macrophages

Flavonoid	Inhibition (%)[a]		
	Concentration (μM)		
	10	50	100
1	11 ± 5	63 ± 1	79 ± 2
2	91 ± 2	99 ± 1	100
3	−12 ± 4	−14 ± 6	−19 ± 6
4	−4 ± 1	74 ± 4	78 ± 3
5	86 ± 1	98 ± 1	100
6	−4 ± 2	55 ± 2	82 ± 8
7	41 ± 2	ND	43 ± 3
8	49 ± 9	96 ± 1	98 ± 1
9	92 ± 1	99 ± 1	100
10	30 ± 7	32 ± 8	37 ± 9
11	93 ± 1	100	100
12	95 ± 1	100	100
13	98 ± 1	100	100
14	29 ± 4	86 ± 1	88 ± 1
15	56 ± 10	88 ± 1	90 ± 1

[a]The rates of CL produced by 1×10^6 macrophages for 20 min is the absence of flavonoids were 85000 ± 2000 (integral counts). A negative value indicates stimulation. Results are expressed as mean ± SD, n = 3–5.
ND: not determined.

of secreting an array of cytotoxic products that include ROIs and RONIs.

The adherent populations from mouse peritoneal cavities were used as macrophage models in this study. Thioglycollate – elicited peritoneal macrophages from BALB/c mice released significant amounts of nitrite upon stimulation with Con A (*in vitro*) and LPS (*in vitro*). This model for activation of macrophages has been reported in our previous study (Król *et al.*, 1995a).

Thioglycollate elicited and Con A + LPS – primed macrophages express cytostatic activity against P815 tumour cells, and release interleukin-6 and tumour necrosis factor-α. Similarly, activated macrophages secrete of ROIs (Król *et al.*, 1995b)

We have shown here that flavones and isoflavones inhibit production of nitrite, a chemical product of NO. These compounds also inhibit luminol-dependent chemiluminescence. The cellular mechanism of the effects are not clear, but may by related to known biological effects of the flavones, such as antioxidant and antiradical properties (Robak & Gryglewski, 1988; Cotelle, Bernier, Catteau, Pommery, Wallet & Gaydon, 1996), inhibition of oxido-reductases (Tauber, Fay & Marletta, 1984) and inhibition of cellular enzymes involved in a signal transduction (Cunningham *et al.*, 1992; Cushman, Zhu, Geahlen & Kraker, 1994). 3'-Amino-4'-hydroxyflavone and 3-hydroxyflavone was the most potent inhibitors of NO production in murine macrophages and were evaluated for inhibition of the generation of ROIs by macrophages. In a previous study from this laboratory we demonstrated that the flavonols (3-hydroxyflavone derivatives) also was the most decreased neutrophil chemiluminescence (Król, Shani, Czuba & Scheller, 1992; Król, Czuba, Scheller, Paradowski & Shani, 1994). Interestingly, the concentration of release of NO by the macrophages closely match these values. These data strongly suggest that production of NO is a major controlling factor in generation of chemiluminescence upon activation of the macrophages. Also these results show that the flavones can modulate the immune responses and the inflammatory reactions by controlling productions of NO.

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Macromolecular Systems for Chemotherapy and Magnetic Resonance Imaging

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Macromolecular systems for chemotherapy and magnetic resonance imaging

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Abstract

The potential of macromolecular pro-drugs in drug delivery and diagnostic imaging lies in their ability to modify the pharmacokinetic distribution of low molecular weight drugs or diagnostic agents. At a simple level this may provide a means of sustaining release of drug from a soluble macromolecule which is retained in the circulation, perhaps avoiding distribution of active drug to a toxicity compartment in the process. There is greater potential in applications which make use of the intrinsic biodistribution of inert macromolecules, in relation to their hydrodynamic radius and net charge. Long circulating macromolecular systems may have advantages in magnetic resonance imaging of the blood circulation and diagnosis of damaged or inflamed tissues, which may take up the macromolecule to a greater extent than normal tissues. A related technology is developing based on the concept that circulating macromolecules accumulate passively in tumours, due to enhanced endothelial permeability and retention of the macromolecule due to poor lymphatic drainage. A secondary advantage potentially could be gained by targeting macromolecules to specific cells by receptor-mediated endocytosis, and designing systems which are degraded by lysosomal enzymes to release active drug in the target cell. The drawbacks of macromolecules are their limited penetration into tissues and the relatively slow rates of internalisation by endocytosis, which have discouraged drug delivery scientists in the recent past. Yet this field is still in its infancy. The tissue distribution of macromolecules with regard to polymer chemistry, molecular weight and charge are not yet fully understood, and advances in this field will depend on the synthesis of well-defined polymers, and careful characterisation of their properties. Here we review the rationale for the use of macromolecules in chemotherapy, the susceptibility of macromolecular pro-drugs to lysosomal degradation, developments in synthetic approaches within the field, and discuss how macromolecular pro-drug chemistry affects their biological properties. We pay particular attention to the rationale for their use in magnetic resonance imaging and the selection of MRI contrast agents for coupling to polymers.

Keywords: Tumor targeting; Lysosomal degradation; Biodistribution; Polysaccharide; Poly(amino acid); Water-soluble polyacrylate; Poly(ethylene glycol); Chelating agent; Paramagnetic ion

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Contents

1. Introduction

The general model of a macromolecular prodrug, first proposed by Ringsdorf in 1975 [1,2] is represented in Fig. 1. The strategy of coupling a drug of low molecular weight to a macromolecule, may result in one or both of the following advantages. Firstly, a controlled release system may be achieved and, secondly, by passive or active means, a targeted system can be designed.

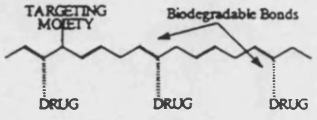


Fig. 1.

Targeted systems may be of benefit for both therapeutic and diagnostic applications.

The macromolecule itself should be non-toxic and non-immunogenic and should be easily manipulated for chemical reactivity. Originally, the macromolecules used for such purposes were naturally occurring; for example DNA [3], albumin [4] and dextran [5]. However, synthetic polymers, which have been previously used as plasma expanders, are now more commonly used. Targeting groups can be easily attached to such polymers either to increase non-specific cellular interactions [6] or to exploit receptor interactions to elicit a specific effect [7]. Drug molecules can either be attached to the polymer via a non-degradable spacer or by degradable linkages [8].

Attachment of a drug to a macromolecule is of particular interest as a therapy for cancer. Macromolecules are known to accumulate passively within solid tumours, giving a first level of targeting [9]. Once a drug is attached to a macromolecule, it can no longer enter a cell by diffusion and must enter by endocytosis which is often enhanced in tumour cells. This advantage can be enhanced by incorporation of specific ligands to produce a second level of receptor-mediated targeting. When access to the cell is limited to receptor-mediated endocytosis, 'lysosomotropic drugs' potentially can be designed such that release of drug only occurs within the lysosomes, thereby conferring a high level of specificity [3].

The disadvantage of such systems lies in the limited penetration of tissues by macromolecules, and their slow rate of cellular internalisation. Improvements in the design of macromolecular pro-drugs are required to optimise their pharmacokinetic distribution, and our understanding of their activity in vivo will be greatly enhanced when they are more clearly defined chemically and better characterised. We do not consider in detail here the large field of antibody-mediated targeting. Antibodies have frequently been coupled to water-soluble macromolecular pro-drugs but a comprehensive survey of this field is beyond the scope of this review. Here we focus on the chemistry of macromolecular pro-drugs, discuss how their chemistry influences their pharmaceutical properties and fate in vivo and pay particular attention to the design of contrast agents for magnetic resonance imaging (MRI). We believe that macromolecules have considerable potential as MRI contrast agents and the information gained by imaging will in turn help our understanding of macromolecular access to individual tissues, and aid the design of systems for therapeutic uses.

2. Uptake of macromolecules into tumours

2.1. Introduction

Many investigators, have demonstrated an increased uptake of macromolecules into tumour cells [10–13]. Originally, this was thought to be due to an increase in the rate of constitutive pinocytosis in tumour cells [10]. However, more recent studies have suggested that the increased uptake may be due to an 'Enhanced Permeation and Retention Effect' (EPR effect) [9].

Matsumura and Maeda [9] investigated the accumulation in tumours of a series of radioactive macromolecules and Evans' blue, a dye. The uptake of a range of polymers of molecular mass 12000–150000 Da was investigated in tumour-bearing mice with respect to the plasma clearance time and the time to reach a fixed gradient (5:1) between the tumour and plasma. Macromolecules of above 16000 Da accumulated in the tumours, all reaching the desired ratio. However, it was apparent from the data that the ideal range for the macromolecules was between 15000 and 70000 Da. The investigators proposed that the accumulation was due to four properties of tumour tissues; increased angiogenesis, enhanced vascular permeability, limited macromolecular recovery via post-capillary venules and lack of functioning lymphatic systems [9]. The rate of angiogenesis in some tumour tissues is greatly increased [11,12] and tumour growth is frequently dependent on the development of a new functioning vascular system. To achieve this increased growth, tumours secrete a range of angiogenic factors, such as the polypeptide angiogenin [9,14,15] These factors either act directly on the dividing cells or stimulate the release of endothelial growth factors from other cells. The increased number of blood vessels can be easily demonstrated using angiography [9].

2.2. Enhanced vascular permeability

Five types of molecular movement through endothelium have been described [13]:

1. Simple passive diffusion of the molecule across the cell
2. Lateral diffusion of the molecule in the cell membrane allowing transport around the cell and across the barrier
3. Passage through inter-endothelial cell junctions
4. Passage through endothelial gaps, i.e., fenestrae or sinusoids
5. Vesicular transport

The first two mechanisms can be discounted when considering the transport of macromolecules, although they are the main methods of transport for small solutes. All of the other mechanisms are involved in the transport of macromolecules. The majority of transport is thought to be based on the formation of water filled pores at the endothelial junctions [13]. The role of vesicles in transport is more widely disputed [11,13]. Vesicular transport can take two forms; the uptake of a macromolecule into a vesicle followed by transport across the cytoplasm and release or linkage of vesicles to form a transient pore across the cell. The importance of the first method is questionable; some workers have suggested it to be the major method of transport of macromolecules, whereas others feel its role is minimal The latter hypothesis is supported by the observations of no change in the rate of transport at reduced temperature where vesicular transport is known to be abolished [11,13].

The transport of macromolecules can be affected by both their size and charge. Measurement of the glomerular filtration rate, an example of endothelial transport, has demonstrated that the hydrodynamic radius of a macromolecule is a more accurate description of the tendency to be transported than is molecular weight [11]. Hence, albumin, a natural macromolecule, has a small radius, due to strong hydrogen bonding, and therefore a large renal threshold, whereas a hydrophobic synthetic polymer with few intramolecular bonds, such as HPMA, exhibits a different renal threshold. The charge of a macromolecule can also affect the transport across endothelial cells. The endothelial surface exhibits a net negative charge and experiments with albumin have shown that cationic albumin is three times more permeable than neutral albumin. This phenomenon is clearly demonstrated in fenestrated endothelia particularly in the renal glomerulus. Most tumours are epithelial and have vasculature with properties different from normal tissue. The endothelium in all but a few cases is continuous having tight endothelial junctions and an intact basement membrane, unlike inflammatory tissue. However as accumulation of macromolecules occurs, the endothelium must be more permeable than normal continuous endothelium [11]. In a very few tumours, angiogenesis is altered, the new blood vessels produced being composed only of endothelial cells, whereas most vessels also contain pericytes. Pericytes are thought to be involved in the maturation of vessels and it has been postulated that lack of pericytes can lead to incomplete basement membranes. This alteration in basement membrane could lead to a non-specific 'leakiness' of cells, allowing accumulation of macromolecules. However, in the majority of cases, no morphological differences in the blood vessels can be observed. This has lead to the hypothesis that tumour cells secrete factors which alter the permeability of endothelium to macromolecules.

Senger et al. [15] have investigated the uptake of labelled human serum albumin and colloidal carbon in tumour bearing guinea pigs. Accumulation was demonstrated in the regions around the tumour and within the tumour. Analysis of the ascitic fluid from the animals and tissue culture experiments allowed the isolation of a protein, which has been called the vascular permeability mediator (VPM). The isolated factor caused increased permeability in cutaneous tissues without causing endothelial cell damage [11]. This protein of between 34000 and 42000 Da has been isolated from a range of tumour cells [16]. These workers have suggested that this factor is released to allow the passage of fibrin into the interstitium to allow coagulation, which is beneficial to tumour growth and angiogenesis [17]. A second factor, bradykinin, has also been investigated as a promoter of tumour permeability [13]. Increased concentrations of both bradykinin and Hyp^3-bradykinin have been found in numerous tumour types including stomach, pancreas and ovarian cancer [13]. Bradykinin is known to have both permeability and pain-inducing properties but it also is involved in the activation of phospholipase A_2, leading to the formation of another permeability factor, prostaglandin E_2. Other permeability factors have been investigated. Obvious candidates are the leukotrienes, which have been found in increased levels in most inflamed tissue but not, as yet, in tumour cells. Other potential permeability enhancers include tumour necrosis factor (TNF),

platelet derived growth factor (PDGF), serotonin, interleukin 2 and leukokinin [16].

2.3. Macromolecular recycling and accumulation in tumours

Once molecules are present in the interstitium, they can be taken into cells of the organ or passed back into the blood stream. Small molecules generally pass easily into the blood stream via the post-capillary venules, as described earlier. The endothelial cells of these contain slightly enlarged inter-endothelial junctions. However, these spaces are too small for the passage of macromolecules. Natural macromolecules [11–13,16] pass into lymph capillaries, which are formed from capillaries but have very large endothelial spaces. Lymphatic capillaries form a circulation allowing the movement of macromolecules away from an organ. The macromolecules are eventually passed from the lymphatic system into the thoracic duct which empties into the blood stream via the sub-clavian vein [9,11]. Macromolecules of lipid nature are particularly attracted into the lymphatic system. Experiments involving Lipiodol, an iodinated derivative of poppy seed oil, have shown that it is selectively taken into lymphatics and 'recycled' via the lymphatic system. However when Lipiodol was injected into a tumour-feeding artery [18] it was accumulated in the tumour. This suggests that tumours have little lymphatic drainage. This proposal has been confirmed by imaging experiments which have shown little lymphatic development in tumours [9,11]. Macromolecules in tumour tissues can therefore only escape by slowly diffusing through the tumour mass to the surface where they can be recovered by the well-developed lymphatic system of surrounding organs. This transport is minimal, as macromolecules are too large to diffuse rapidly [19]. The macromolecule accumulates and a depot of 'drug' is formed. This combination of enhanced permeability and lack of lymphatic drainage results in an highly effective 'passive' targeting of macromolecules and hence macromolecular prodrugs to tumour cells.

2.4. Modulation of the enhanced permeability and retention (EPR) effect

Following the identification of the EPR effect, Maeda and colleagues have investigated ways of manipulating the properties which lead to enhanced uptake to allow even more effective chemotherapy using macromolecular pro-drugs. Bradykinin exhibits an effect on the vascular permeability either directly or through prostaglandin-E_2 [13]. It is formed by the action of kallikrein on kininogen and is continually broken down by kinase I and II enzymes (Fig. 2). Inhibition of kinases I and II will lead to increased concentrations of bradykinin and hence to increased vascular permeability. These enzymes are inhibited by inhibitors of angiotensin-converting enzymes (ACE), e.g., captopril and enalapril [12]. Concurrent administration of an inhibitor with a macromolecule would lead to enhanced uptake of the macromolecule into the tumour, whilst normal tissue would remain unaffected as the kinin cascade would not be in operation. Investigations on the uptake of labelled albumin into a mouse ascitic tumour in the presence of such an inhibitor (10 mg kg^{-1} enalapril) showed a 50% increase [12].

In a few tumours, this exploitation could prove disastrous, as macromolecular influx also results in fluid accumulation which can be difficult to control. In these cases, administration of soybean trypsin inhibitor, which halts the cascade, would be more beneficial [12]. Several tumours have a hypervasculature allowing increased blood flow to the tumour. These blood vessels have been shown to be unaffected by angiotensin II. This is thought to be due to a lack of contractile smooth muscle in the blood vessels

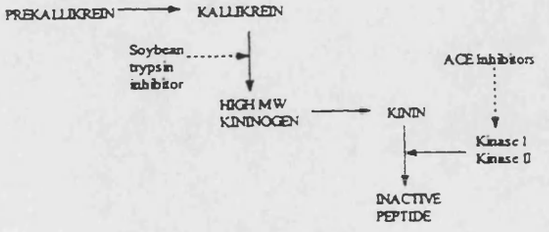


Fig. 2.

rather than to a lack of receptors for angiotensin II [13]. Thus, if a hypotensive state were to be produced in a patient, the junctions between the endothelium would be tightened and less passage of macromolecules would occur. However, in tumour vasculature, the vessels would remain as normal, allowing passage of macromolecules. Clinical data have been produced using this method with small molecules. Both an increased regression of tumour size and improved response rate were demonstrated. It is thought that, with small molecules, once the hypertension wears off, back-flow of the molecules can occur; this would not be the case with macromolecules. Thus, studies with macromolecules [16] have shown a 2-fold concentration enhancement in tumours 6 h after the hypotensive event, as well as decreased levels in the bone marrow. Unfortunately, this approach is of little value in naturally hypertensive patients.

3. Cellular uptake of macromolecules

3.1. Mechanism of internalisation

Small solutes are easily taken into cells via passive diffusion or through the action of specific porters for certain substrates such as glucose and amino acids [20]. Macromolecules, however, cannot enter a cell by such a route because of their size. Natural macromolecules such as proteins, polysaccharides and polynucleotides are taken into the cell by endocytosis. Synthetic macromolecules also follow this route. Internalisation can be considered to occur by one of two clearly different mechanisms; pinocytosis or phagocytosis. Phagocytosis or 'cell eating' occurs only in specialised cells such as macrophages [21] and can result in the uptake of particles larger than 10–20 μm in diameter. It is of major interest in the immune system, as it is involved in the uptake and destruction of bacteria. This process is of minor importance when considering water-soluble macromolecular pro-drugs but can be significant in approaches to drug targeting using liposomes and microparticles.

Whereas phagocytosis occurs only in response to a presentation event [22], pinocytosis is a constitutive phenomenon in all mammalian cells [21]. It has been likened to drinking as it involves the continual uptake of extracellular fluid. Pinocytosis is also known as fluid phase endocytosis. The initial endocytic event in pinocytosis is the formation of an invagination in the plasma membrane. A vesicle (pinosome) is then formed encapsulating extracellular fluid. This then buds away from the cell surface into the cytoplasm and then fuses with other pinosomes within the endosomal compartment without release of the contents. The default path of the macromolecule is vesicular transport deeper into the cell and subsequent vesicular fusion with a lysosome (a degradative vesicle released from the Golgi Apparatus) to form a secondary lysosome. Within the secondary lysosome, degradation of macromolecules occurs and small molecules are released into the cytoplasm. The secondary lysosome may then form a residual body, which contains non-degradable material and remains in the cell for its lifetime or vesicles which can fuse with the cell surface allowing exocytosis of the contents [11,20,22,].

Each cell has a constitutive rate of pinocytosis which can be measured by the use of polymers such as inulin, polyvinylpyrrolidone (PVP) [22] or poly(hydroxypropylmethacrylamide) (HPMA) [23,24] which show no specific adsorption to the cell surface. In this case, uptake by fluid phase pinocytosis is directly related to the concentration of the 'probe' in the medium. The rate of uptake is expressed as the endocytic index – 'the volume of the culture medium which is captured in a defined number of cells in a defined time' [25]. This allows comparisons to be made on the uptake of various macromolecules and on the differing rates of uptake in different cell lines. The endocytic index also allows the measurement of three other types of pinocytosis, non-specific adsorptive pinocytosis, receptor mediated pinocytosis and 'piggy back' endocytosis [25]. Measurement of the endocytic index for some macromolecules shows an increase over the model polymers. Two hypotheses for this event have been put forward. Firstly, the macromolecule is taken up by fluid phase pinocytosis but exerts a pharmacological effect on the cell, leading to an increased rate of constitutive

pinocytosis. This hypothesis can be refuted easily by measuring the rate of uptake of a fluid phase 'probe' in the presence of the test macromolecule. If the rate of uptake of the 'probe' is unaltered, the test macromolecule cannot have been taken up by fluid phase pinocytosis [25]. The second hypothesis is more likely, that the macromolecule is internalised faster owing to adsorptive endocytosis, that is, a direct interaction with the cell surface resulting in enhanced uptake into endocytic vesicles. There are two types of adsorptive pinocytosis; non-specific – due to changes in hydrophobicity or charge, and receptor-mediated pinocytosis – where specific interactions between receptor and substrate occur [22].

Interaction of a ligand with a receptor may lead to a more organised pathway of internalisation. Firstly, the ligand binds to receptor sites which are spread over the surface of the cell. Clustering of the receptors then occurs on the cell surface in areas called coated pits. These areas have a protein, clathrin, associated with their surface. This and other associated proteins have roles in causing formation of vesicles and budding off to form coated vesicles which pass the ligand to the endosome for further processing, whilst in some circumstances allowing recycling of the receptor-clathrin complex to the cell surface via a recycling vesicle. It has been suggested that the clathrin does not enter the intracellular regions; rather, uncoated vesicles containing receptor and ligand (receptosomes) are passed into the cell for processing [22,26]. Receptor-mediated endocytosis can be a rapid means of uptake due to specific mechanisms but some receptors need not be internalised by special means and are probably internalised as a result of general membrane turnover in constitutive endocytosis. Under these circumstances, the uptake of a bound ligand may take place at a rate similar to that observed in non-specific adsorptive endocytosis.

The term 'piggy-back' endocytosis has been used to describe enhanced uptake of one molecule due to its binding to another molecule [27]. It is a process which could be considered to be exploited by all macromolecular pro-drugs, in that the coupling of a small molecule to a macromolecule restricts its uptake to endocytosis and, hopefully, leads to reduced non-target interactions [3]. An early example of the use of 'piggy-back endocytosis' to elicit a therapeutic effect was demonstrated by Trouet et al. [3]. Ethidium bromide is active against *Trypanosoma cruzi* in vitro but is inactive in vivo. In host organisms, the parasite lives within lysosomes where it is unharmed by lysosomal enzymes. It is protected from ethidium bromide, since this is a small molecule which passes into cells by diffusion and cannot pass into lysosomes. Attachment of ethidium bromide to DNA results in death of the parasite as the conjugate is taken up by pinocytosis and can act within the lysosome. This process may well explain many early results which suggested that enhanced rates of uptake of macromolecules were due to enhanced rates of endocytosis in tumours. For example, the uptake of colloidal radioactive gold is enhanced in the presence of poly(L-lysine). This effect is more likely to be due to association of the polycation with the cell membrane, so that the macromolecular complex is taken up by non-specific adsorptive pinocytosis rather than by an increased rate of formation of endosomes [27].

3.2. Factors affecting rate and mechanism of internalisation

Early experimental work [25] demonstrated an increased rate of uptake of denatured bovine serum albumin over untreated albumin in rat yolk sacs. As the rate of formation of pinosomes was unchanged, these results were attributed to an increased affinity for the cell surface, leading to an increase in non-specific adsorptive pinocytosis. This increase was dependent on the extent of denaturation but was unaffected by the method of denaturation. The researchers believed that this increase was due to either the unmasking of hydrophobic groups or to masking of charged residues [25]. With other denatured proteins such as insulin, uptake was either unaffected by the process or was reduced owing to decreased binding. Thus, it was proposed that binding sites both for hydrophobic regions and for charged regions exist in yolk sacs. Polypeptides are very difficult to study in this context,

since each amino acid could potentially exert a profound affect on the binding capabilities. Experiments with model polymers, however, give more reliable data on cell surface interactions. Both PVP and HPMA have little or no affinity for the cell surface and are taken up by fluid phase pinocytosis; hence, they are good models for investigating the effect of hydrophobic groups.

Duncan et al. [6] have demonstrated in vitro that the incorporation of tyrosinamide residues onto HPMA leads to an increased uptake in rat yolk sacs. The effect was dependent on the molar percentage of the hydrophobic moiety, the highest percentage incorporation (15.4 mol%), resulting in a 10-fold increase in the endocytic index. Interestingly, the effect was not seen below a threshold level of 10 mol% and the authors suggested that this was due to a requirement for hydrophobic domains before non-specific binding occurs. Similar results have been reported using poly(α,β-(*N*-(2-hydroxyethyl))-DL-aspartamide) (PHEA) [28–30]. Incorporation of a tyramine derivative at a range of concentrations between 1.2 and 21.9 mol% resulted in enhanced uptake above a threshold value of 10 mol%. The increase was due to a non-specific interaction, since, if PHEA and PVP were co-administered, no increase in the rate of uptake of PVP could be measured. Endocytosis can only be enhanced by such a mechanism if the hydrophobic moieties are exposed and therefore available for interactions with the cell surface [27]. Studies on a block co-polymer of poly(ethylene glycol) (PEG) and poly(L-lysine) (6:3) substituted with highly hydrophobic palmitoyl derivatives demonstrated no increase in uptake of the co-polymer over that of PEG alone [31]. This initially seems surprising. However, further evaluation of the co-polymer revealed that the polymer forms a micelle in solution in which the hydrophobic groups are in the core and the less hydrophobic PEG is exposed to the cell surface. It should be noted that PEG alone does show an increased uptake in comparison with PVP, demonstrating a level of hydrophobic non-specific interactions.

The effect of charge is more complex; both anionic and cationic polymers exhibit characteristics of adsorptive pinocytosis. Co-polymerisation of a vinylamine with PVP (8 mol%) results in a cationic polymer which has increased endocytic activity in both rat macrophages and yolk sac [32]. Equally, the anionic polymer, DIVEMA pyran-co-polymer, is taken up by a non-specific adsorptive process [20], though cationic polymers usually exhibit a more pronounced adsorption and faster rate of internalisation. The effect of size and molecular weight on macromolecular uptake is more properly defined as the effect of the hydrodynamic radius of the polymer. This is affected by the ability of the polymer to form intramolecular hydrogen bonds or van der Waals interactions. The size of the pinocytic vesicles is the main barrier to uptake and varies according to cell type. Ease of access to the cell surface also requires consideration [11]. Studies on both HPMA [27] and PVP [32] have shown the uptake to be size-dependent in the rat yolk sac. Co-polymers of PVP and vinylamine of molecular mass 120000 Da were taken up slowly in comparison to those of molecular mass 46000 Da. In comparisons using rat macrophages, uptake of the larger polymer was more efficient.

All the modifications above result in an increase in non-specific interactions; however, many cells also possess receptors for particular substrates on the cell surface. These receptor-substrate interactions can be exploited to allow the delivery of macromolecules to specific cell lines. Such receptor-substrate interactions include carbohydrate-lectin, antibody-antigen and hormone-receptor interactions. The area of lectin-mediated drug delivery has been widely explored [3,33]. Many cells express lectins on their surface and most are limited to one cell type [37]. Substrates for lectins are, however, more diverse, although many do show structural similarities. In vivo, lectins bind complex oligosaccharides. Therefore, it is possible for one lectin to bind two structurally different sugars in different areas of the binding sites. An example of this is the P-selectin on endothelial cells which binds the oligosaccharide Lewis^x which contains both galactose and fructose. Polymers containing

either galactose or fructose can also bind to the lectin [37]. As lectins bind oligosaccharides, it has recently been suggested that triantennary targeting ligands should be attached rather than single sugar molecules. Experiments with the asialoglycoprotein that binds *N*-acetyllactosamine have shown that binding of an oligosaccharide containing three *N*-acetyllactosamine antennae is higher than those containing one or two [33]. This asialoglycoprotein is expressed in many but not all hepatocyte cells. The receptor is present on the venous face of hepatocytes, allowing for interactions with blood-borne substrates. The receptor consists of at least two sub-units, each capable of binding one galactose residue, raising the possibility of multi-ligand binding [33]. Hepatocytes are valuable target cells for many diseases such as hepatitis [26]. The potential of targeting to hepatoma cells, however, is low. Most tumours in the liver are either primary hepatomas or metastases of breast or colo-rectal cancer. The metastases comprise modified cells from the region of the primary tumour, so do not contain the asialoglycoprotein [26]. In primary tumours, expression of the asialoglycoprotein is sometimes retained but appears to be cell-line dependent. The degree of expression is also often reduced when compared to basal levels in hepatocytes. This suggests that organ-specific delivery is possible but that tumour cell-specific delivery is unlikely by this mechanism [26,33].

The hepatocyte asialoglycoprotein has been targeted using a number of polymers bearing galactose residues both in vitro and in vivo. Vansteenkiste [34] investigated the distribution of dextran containing one (6 mol%) or three D-galactose residues (8.5 mol%) in mice in vivo. 30 min after injection, the triantennary dextran displayed the highest uptake into liver (71%). The polymer containing only one residue and the unmodified dextran accumulated less (43 and 16% respectively). The increase in the uptake of the triantennary containing polymer could be due to improved binding but other workers [33] have suggested that the effect is due to the larger mol% of galactose residues. Another possibility is that the polymer is being picked up by a different receptor, the Kupffer cell 'galactose particle' receptor [33,37]. HPMA co-polymers bearing galactose have been evaluated in both rats and mice [14,23,35,36]. The kinetics of the galactose-substituted polymers displayed a more rapid clearance from blood and an increased uptake into the liver. In studies in vitro using the $HepG_2$ cell line, a human tumour cell line known to express the asialoglycoprotein receptor, a 7-fold increase in uptake was observed using the galactose-containing polymer [23].

An interesting model to test the feasibility of drug targeting is the fucose receptor on L1210 mouse leukaemia cells [33,37], which has been investigated by Duncan et al. [36]. A conjugate of HPMA bearing the cytotoxic drug sarcolysin was further derivatised by the inclusion of fucosylamine. The fucosylamine derivative caused greater cytotoxicity in vitro than did the underivatised polymer. A second HPMA co-polymer bearing daunomycin and fucosylamine has also been tested both in L1210 and CCRF cells (a human leukaemia cell line which does not have fucose receptors). Inclusion of fucosylamine enhanced the inhibitory effect of daunomycin in the L1210 cells but not in the CCRF cells. Similar polymers containing galactosamine rather than fucosylamine were no more effective than underivatised polymers [38]. These polymers have also been investigated in vivo in DBA_2 mice inoculated intraperitoneally with L1210 cells. In this situation, incorporation of the carbohydrate gave no significant advantage on day 1 after inoculation. After 3 days, however, the fucosylamine-coupled HPMA was more effective than control polymers lacking this moiety. It has been suggested that this may be due to the increased number of fucose receptors arising from rapid proliferation of the tumour cells [36]. Studies in vivo using HPMA bearing fucosylamine and adriamycin gave similar results. Again, the conjugates containing fucosylamine were more effective than those without, especially at low doses. Unfortunately, an increased liver concentration of these polymers was seen, along with targeting to the tumour. This may be due to other cells, such as hepatocytes and macrophages [39], having fucose-recognising receptors. Interestingly, attempts to increase cellular interactions

using triantennary ligands have been unsuccessful [40], although this may have been due to a lack of flexibility in the spacer employed.

Macrophages are not an obvious target cell for chemotherapy. However, delivery of a macrophage-activating agent such as *N*-acetylmuramyl dipeptide (MDP) to the macrophages could result in increased phagocytic activity against tumour cells and metastatic cells in particular [37]. Many sugars interact with this receptor but are of two main classes, one based on D-mannose and one on L-galactose [33]. Studies involving both glucosamine and mannosamine HPMA glycoconjugates demonstrate little change in kinetics at low levels of substitution. More highly substituted polymers were accumulated in the liver, possibly owing to interactions with Kupffer cells [41]. Monsigny and colleagues have investigated poly(L-lysine) bearing both MDP and mannose-related sugars and were able to demonstrate activity in activating macrophages [37]. The studies reported thus far demonstrate the potential of lectin-targeting in enhancing chemotherapy. However, more investigation of the lectins expressed by tumour cells is required before such targeting becomes a clinical reality.

Over the past few decades, much interest has been focussed on exploiting the specific antibody-antigen interaction as a means of targeting drugs to particular cells [1]. This approach, however, has been beset with difficulties such as immunogenicity [52] and poor systemic distribution in vivo. Also, the realisation that tumours are distinctly heterogeneous and thus display various numbers of antibody-binding sites per cell, within the same tumour, has led to a reduced interest in antibody targeting. Using only one antibody conjugate is unlikely to allow interactions with every tumour cell. Despite these problems, a number of macromolecular conjugates have been developed and tested [43,44]. Much interest has been focussed on antibodies to the transferrin receptor [42,45] which is present on most proliferating cells but is expressed in high numbers on tumour cells [45]. Studies in vitro using the monoclonal antibody B3/25 attached to HPMA demonstrated one of the main difficulties in using antibodies. The conjugate was no more actively internalised than one containing non-specific IgG, showing that conjugation had eliminated the specificity of the antibody [42]. Other workers [46–50] have examined targeting of co-polymers bearing anti-Thy 1.2 antibodies which interact with Thy 1.2 alloantigens on mouse splenic cells. From these studies, it became apparent that the coupling method used was of great importance in retaining activity [50]. The targeting of HPMA copolymers to colorectal cancer has also been investigated, using Fab' fragments of specific antibodies [51].

An alternative approach was investigated by Shen et al. [52] who developed a conjugate of poly(D-lysine) where the antibody was bound via the Fab regions to expose only the Fc region. As many cells exhibit Fc receptors, the polymer can act as a hapten for interactions. Many tumour cells show Fc receptors, including a few which are derived from cells which do not have this receptor type. This last type are of particular interest in drug targeting. The polymer was taken up more effectively than with non-targeted polymers in Fc-positive cells but, as expected, showed no enhancement in Fc-negative cells.

A further consideration is the use of hormone receptor interactions to promote internalisation and drug targeting. The melanocyte stimulating hormone receptor (α-MSH receptor) is of interest, as this receptor is expressed by the majority of melanocytes in malignant melanoma [53]. Malignant melanoma is a cancer which is difficult to treat, often leading to hepatic and brain metastases. Effective targeting of the HPMA polymer was demonstrated both in vitro in B16F10 cells, a cell line which exhibits α-MSH receptors, and in vivo in mice inoculated with B16F10. Binding of the hormone to the polymer through its N-terminus did not affect activity. This is not surprising, as the receptor binding site of this tridecapeptide is the region between the sixth and tenth residues which are unaffected by tion to the polymer. Although these results are promising, it has been reported that other receptors for MSH are expressed in other tissues, particularly in the brain [54,55], which may limit the therapeutic opportunities for these conjugates, unless a high level of selectivity can be achieved.

4. Lysosomal degradation

Once a drug molecule is only able to pass into a cell by endocytosis, as in the case of a macromolecular pro-drug, the default destination of that macromolecule is pre-determined to be fusion with a lysosome. Lysosomes are the organelles of cells responsible for digestion and are ubiquitous in living mammalian cells [20]. The lysosomal membrane is impermeable to macromolecules and most initial products of digestion such as oligosaccharides. However, it is freely permeable to single units such as amino acids, monosaccharides and mononucleotides. The lysosomal membrane also contains specific porters for some amino acids to allow rapid transport of the degradation products to the cytoplasm [56]. Lysosomes exhibit unique characteristics in terms of pH and enzyme activity. Lysosomal pH is slightly acidic, usually about pH 5.5, in comparison to serum [26]. This is an important consideration as the drug delivered should not be acid sensitive. Lysosomes contain over fifty digestive enzymes, the majority of which are hydrolytic in nature. They allow the cleavage of all common natural inter-monomer bonds such as peptidyl, glycosyl and phosphate linkages. One family of enzymes found in lysosomes are of particular interest, the cathepsins. These thiol-proteinases cathepsin B, H, and L are involved in peptide cleavage, as are the non-thiol dependent cathepsin C, a peptidyldipeptidase, and cathepsin D, an endopeptidase [57].

These characteristics of lysosomes afford the means of selective release of active drug from its macromolecular carrier within the cell, conferring the highest level of targeting of drugs. Release of the drug can be achieved by exploiting the acidic pH of lysosomes, the specific and high level enzyme activity or the reductive properties of the endosomal pathway [3]. Many workers have shown that release of a drug from a macromolecule is better achieved by incorporating a spacer unit between the macromolecule and drug [8]. Otherwise, the macromolecule itself must be degradable, a possibility which is discussed below. The ideal properties of the linkage are that it should render the drug inactive, that it should be stable in serum and that it be readily broken down in lysosomes. Both covalent and non-covalent linkages are acceptable. However, a non-covalent linkage, although easier to form, is unlikely to be as stable in serum as is a covalent linkage [3].

Shen [58] first explored the potential of acid-labile spacers in 1981, using a cisaconityl acid spacer between daunomycin and poly(D-lysine). Conjugates bearing this linkage demonstrated 90% growth inhibition of WEHI-5 cells, whereas polymers with *N*-maleyl spacers had no effect (Fig. 3).

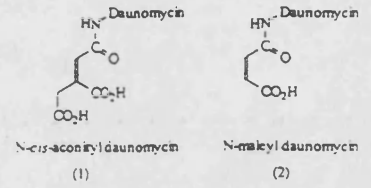
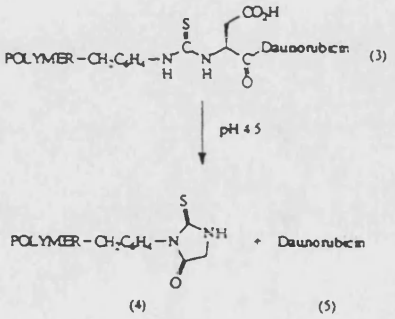


Fig. 3.

A further example of acid-labile spacers are those based on *p*-benzylthiocarbamoyl groups (Scheme 1) [59]. In slightly acidic conditions, as in a lysosome, this spacer undergoes an Edman cyclisation, resulting in release of daunomycin. This is only achieved if the amino acid used is acidic, such as aspartic acid. Studies comparing the side-arm attached to aspartic acid or leucine demonstrated the suitability of the linkage. With aspartic acid, no drug release was seen in serum over the 3 weeks of the study but release was afforded at pH 5 and pH 4. The leucine derivative did not undergo cyclisation to form a thiazolinone at pH 7.4 or pH 5 and only a small amount of release was seen at pH 4 [59].



Scheme 1.

More recently, Shen [60] has suggested that acid-labile drug arms are relatively unsuitable as the difference in pH between serum and lysosomes is not large enough to provide totally site-specific degradation. In a novel approach, he has investigated the potential of non-covalent acid-sensitive linkages. The receptor-substrate complexes on the surface of cells are robust but are easily broken down in lysosomes. This is due to a conformational change in the receptor at the lower pH resulting in a decreased binding affinity for the substrate. Shen has developed a polymer poly(glutaminylhistamine-glutamate) (PHG) which forms a pH-dependent complex with poly-(lysine). Release of poly(lysine)-methotrexate from PHG was demonstrated in lysosomes [60]. Obviously, further work is required on this approach before it can be utilised.

Degradation of proteins is often achieved by the reductive cleavage of disulphide bonds and it is thought that this reaction is the first step in the degradation of insulin. The actual location of this reaction is unknown but it is believed that it occurs early in the endosomal pathway, as shown by studies on diphtheria toxin. Diphtheria toxin consists of two sub-units A and B linked via a disulphide bond. Release of the active B portion is afforded by cleavage of the bond but, as this portion of the toxin is acid-sensitive, this cleavage and release must occur before reaching the lysosome. Shen et al. [8,52] have investigated disulphide bonds for release of methotrexate from poly(D-lysine). Poly(D-lysine) is an ideal choice of polymer, as it is not degraded by lysosomes and any release of drug must result from cleavage of the disulphide bond. The conjugate was effective in both normal and methotrexate-deficient cells and was unaffected by leupeptin or decreased glutathione concentrations; however, its activity was abolished by 2-mercaptoethanol. These results demonstrate that cleavage does not occur on the cell surface, is independent of enzymes and acidic pH and is not a non-enzymatic reaction with glutathione, confirming the view that the reduction occurs in pinosomes prior to fusion with the pre-lysosome. More recently, Bonfils et al. [61] have used disulphide bonds to link oligonucleotides to mannosylated proteins. These conjugates were stable in incubation with serum but rapidly released the nucleotide within cells.

Most interest in the development of spacers has been focussed on amino acid sequences which are specific to lysosomal degradation by enzymes. One of the earliest examples of this approach dates from 1954. Jatzkewitz employed a dipeptide sequence GlyLeu to attach mescaline to poly(vinylpyrrolidone-co-acrylic acid). This increased the biological half-life of mescaline from a few hours to 17 days [62]. The specificity of lysosomal enzymes can be described using the method of Schechter and Berger (see Ref. 63). The substrate specificity of many enzymes is now known, enabling the design of spacers for testing.

Duncan, Kopecek, Ulbrich and colleagues, over a 15 year period, have made a made a major contribution to our understanding of the lysosomal degradation of macromolecular pro-drugs. Initially, pro-drugs based on HPMA were synthesised to contain peptide sequences with known specificity for chymotrypsin to investigate the effect of the macromolecule, the length of spacer and the stereospecificity of the amino acids off the spacer on specificity. The suitability of the sequence was measured by the release of a drug model, *p*-nitroaniline. The binding site of chymotrypsin displays a preference for an amino acid which can act as a hydrogen bond donor in the P_1 position, one able to form van der Waals interactions with Ile^{99} at P_2 and hydrophobic amino acids at P_4 [63]. These specificities were retained where the peptide bond to be cleaved was between a macromolecule and a drug molecule, revealing major differences in the release of drug from spacers of the same length. With smaller spacers, for example those with only two amino acids, substrate specificity was of little importance. In such a case, the polymer backbone occupied some of the binding site, so decreasing the strength of binding. The degradation was also affected, not surprisingly, by the inclusion of unnatural D-amino acids such as D-Phe and D-Ala. In these cases, degradation was greatly decreased [63,64].

The effect of the macromolecule on enzyme action has been further investigated using PVP and PEG [65,66]. Conjugates were synthesised with the same peptide sequences as previously

produced for HPMA and the degradation rates were measured. The same relative degradability of the sequences was seen in all three series; however, the rates of degradation of a sequence varied considerably. The highest rates of release were seen in the PEG series, in that even a spacer of one amino acid was degradable. It is thought that this may be due to enhanced interactions between PEG and the enzyme. PEG is a linear polymer with the ability to form hydrogen bonds (Fig. 4). Ulbrich et al. [66] have proposed possible interactions between PEG and the enzyme binding site.

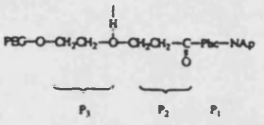


Fig. 4.

A higher rate of degradation was also evident with PVP, although this was not as impressive as that of PEG [65]. This is likely to be due to an interaction of the cationic chymotrypsin with the negatively charged polymer. These studies also provided evidence that increased levels of substitution lead to decreases in degradability. This is probably caused by decreased enzyme accessibility and suggests that there may be an optimum level of side-chain incorporation [65]. Based on this early work, attempts were made to design peptide linkages which are substrates for particular lysosomal cathepsins [57]. Studies were performed using a wide variety of single enzymes and mixed lysosomal extracts but the most promising results were achieved in studies on cathepsin D. Cathepsin D is an endopeptidase with specificity for the bond between hydrophobic amino acids [57]. On the whole, it has little activity with low molecular weight substrates; however, GlyPheLeuGlyPhe is a known substrate. Three sequences were prepared, GlyPheLeuGlyPhe-NAp, GlyGlyPheLeuGlyPhe-NAp and GlyPheLeuGly-NAp and all degraded easily in a mixture of lysosomal enzymes. The first two sequences yielded Phe-NAp. This is not ideal, since release of unmodified drug would ensure target activity whereas amino acid conjugates may prove to be inactive. In the final sequence, however, linear release of unmodified drug was achieved. Interestingly, although this sequence was designed for degradation by cathepsin D, it also fits the substrate conditions for cathepsin L activity, i.e., hydrophobic amino acids in positions P_2 and P_3, and is degraded on incubation with cathepsin B [67,68]. Analysis of the degradation events showed that the terminal -NAp bond was cleaved first to reveal a tetrapeptide which was further broken down to release LeuGly; the final amino acids Gly and Phe were not cleaved from the polymer [57]. These studies provided GlyPheLeuGly as a front-line candidate for a lysosomally degradable sequence. The stability of HPMA co-polymers bearing this sequence in serum was determined. Serum contains a wide range of enzymes including small amounts of lysosomal enzymes which have leaked from cells, although most of the enzymes have trypsin-like activity, i.e., they cleave bonds following Arg and Lys. The co-polymer was not degraded in serum and so was evaluated further [69].

Macromolecular pro-drugs were then prepared incorporating this sequence or the sequence GlyGly which is not degraded by lysosomal enzymes. Numerous drugs, including daunomycin, adriamycin [70], sarcolysin [68] and melphalan [72], have been attached to this sequence and, in all cases, the degradability of GlyPheLeuGly is maintained in lysosomes whilst being minimal in serum. It has also been shown, by the activity of the conjugates, to be degraded in the lysosomes of many cell lines including B16F10 melanoma cells [74], L1210 mouse leukaemia cells [36,38,39,70], Walker sarcoma cells [72] and the $HepG_2$-hepatoma cell line [23]. However, there are some problems which occur with this sequence. Firstly, it is hydrophobic and, in sufficient quantities, could alter the nature of the macromolecule. This would be especially problematic if a hydrophobic drug were then to be attached [75]. Also, in some systems, release of free drug molecules is not achieved, only amino acid derivatives being released. This is probably due to the macromolecule used, e.g., PHEG [76] and its interactions with the enzyme. Nevertheless as has been mentioned earlier, amino acid pro-drugs could be exported from the lysosome resulting in lack of activity [77].

Originally, Trouet investigated macromolecular drug delivery using DNA as a carrier molecule. DNA is biodegradable, so no spacer moiety was required to afford release of drug [78]. However, the limitations of DNA as a carrier led to the use of albumin [79]. Release of the drug from albumin was not achieved from a direct linkage and spacer units were therefore investigated. Following experiments with small amino acid pro-drugs, the ideal bond for release of daunomycin was found to be one between leucine and the drug and the ideal sequence for enzyme specificity to be AlaLeu [80,81]. Pro-drugs were then synthesised with one of three linkages AlaLeu, LeuAlaLeu, and AlaLeu-AlaLeu [79]. Studies in vitro showed release to be highest from the tri- and tetra-peptide sequences and these conjugates were active in studies in vivo. The conjugates were stable in serum for 24 h and this, in combination with the high lysosomal degradability, has lead to this sequence being used in a variety of situations. De Mare et al. [76] have developed spacer sequences based on AlaLeu to attach mitomycin C (MMC) to PHEG. Two sequences, GlyPheAlaLeu and AlaLeuAlaLeu, were sensitive to lysosomal hydrolysis, resulting in release of MMC, although some LeuMMC was also released. The sequence GlyGlyGlyLeu has also been used as a linker between PGA and adriamycin but, again, some amino acid derivatives are produced, suggesting that the sequence has low substrate specificity [75]. In summary the above results suggest that enzymatically degradable spacers provide the greatest selectivity in degradation and, hence, the greatest potential for targeted drug delivery. At this time, macromolecular pro-drugs incorporating the amino acid sequence GlyPheLeuGly are undergoing Phase II clinical trials in humans.

5. Chemistry of macromolecular systems

5.1. Introduction

The major determinant of the pharmacokinetic characteristics in vivo of a macromolecular pro-drug is the macromolecule rather than the drug or targeting moiety. The ideal characteristics of a macromolecule are [4,82]:

1. Ease of synthesis
2. Ease of chemical modification
3. Water solubility
4. Biodegradability
5. Lack of toxicity
6. Lack of immunogenicity

However, at present, no carrier has been found which has all these characteristics. In this section, the best-studied polymers are evaluated with respect to these criteria and related macromolecular drug conjugates are discussed. Both synthetic and natural macromolecules have been used as carriers. Synthetic polymers are considerably more versatile than non-synthetic polymers. They can be prepared in the desired molecular weight fractions and modified for the attachment of drug molecules. They are generally less immunogenic than natural polymers. Unfortunately, many synthetic polymers are not biodegradable owing to their carbon-carbon backbone. Thus, investigators are presently developing co-polymers of synthetic polymers and natural amino acids which retain the favourable properties of synthetic polymers whilst allowing a limited biodegradability.

5.2. DNA

DNA was one of the first macromolecular carriers investigated. It is an ideal lysomotrophic carrier as it is relatively stable in serum but is degraded in the lysosome. In the early 1970s, Trouet et al. [3] produced complexes of daunomycin and adriamycin intercalated into DNA. These complexes proved effective in studies in leukaemia cells in vitro and in DBA_2 mice inoculated with the L1210 leukaemia. Limited clinical trials were performed in terminal leukaemia patients whose disease was resistant to all forms of therapy. The complex was well tolerated and gave very encouraging results; of twenty cases of non-lymphoblastic leukaemia, twelve resulted in complete remission. Interest in DNA as a carrier has waned since then, possibly because of the instructional nature of DNA

which may become incorporated in a random manner into genomic DNA, leading to unwanted side effects, or due to the relative difficulty of preparing drug conjugates [3,78].

5.3. Albumin

The serum protein, albumin, has also been used as a carrier. It is attractive owing to good biological stability, lysosomal degradability, ease of chemical substitution, low toxicity and with homologous albumin, low immunogenicity. Early attempts to produce a methotrexate derivative of albumin resulted in an inactive conjugate [4]. Later attempts to produce a conjugate of daunomycin, attached via degradable spacers to albumin, were rewarded with therapeutic activity. The conjugates lead to a 200% increase in life-span in mice inoculated with L1210 [4,12,79]. There is a large literature on coupling of cytotoxic drugs to proteins, particularly to antibodies, sometimes directly, sometimes by way of a spacer such as albumin or a polymer. We do not attempt to review this field here, as the chemistry of such compounds is ill-defined and their biological properties depend substantially on the properties of the immunoglobulin.

5.4. Polysaccharides

Many polysaccharides have been used as macromolecular carriers including cellulose and starch [5]. However, dextran and inulin have been most widely investigated. Polysaccharides are attractive as carriers because they are easily chemically derivatised and are of both low toxicity and low immunogenicity.

5.4.1. Dextran

Dextran (6) is the collective name for a wide range of polysaccharides based on α-D-glucose having a predominance of 1,6-α linkages, i.e., there is little chain branching (Fig. 5). These polysaccharides are derived from bacteria and the actual composition is determined by their origin. One type of particular interest in pharmaceutical applications is derived from *Leuconostoc mesesteroides*. In this type, 95% of the chain is unbranched and the remaining 5% is branched,

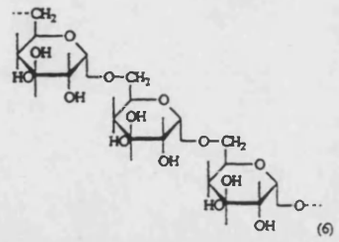
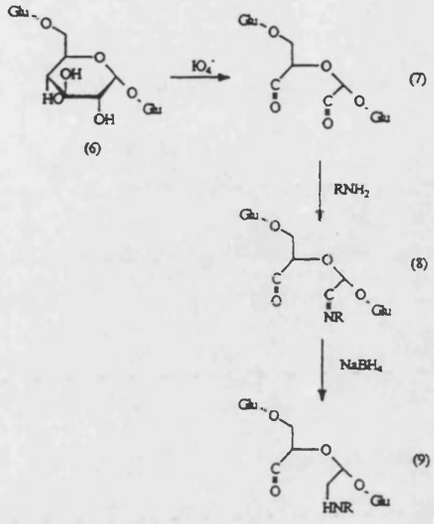


Fig. 5.

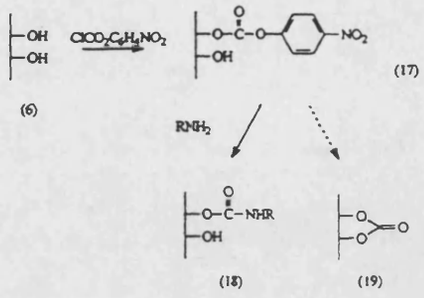
with the majority of the branches only one or two sugars long [5].

Dextran has been used for many years in medical practice. Dextran T-70 (dextran of molecular mass 70000 Da) is used as plasma expander in patients suffering from shock or haemorrhage and dextran T-40 is used to increase blood flow in capillaries in the treatment of vascular occlusion. Thus, from many viewpoints dextran is a good candidate for a macromolecular carrier as its toxic and immunological effects are well characterised. Dextrans are available in narrow molecular weight bands with low polydispersity that allow easy characterisation and evaluation of resulting drug conjugates. They also have very high solubility in water which is retained even with 20% w/w ligand incorporation.

The methods for activation of dextran have been reviewed excellently by Larsen [5] and Schacht [85]. Dextran contains a large number of hydroxyl groups that are suitable either for direct conjugation with carboxylic-acid-bearing drugs such as aspirin or for chemical modification and activation prior to coupling with other drugs. There are three main methods of activation, all of which have been used in the preparation of macromolecular pro-drugs. The most popular method involves the production of dextran dialdehydes by the action of periodic acid (Scheme 2). The resulting aldehydes can then react to form Schiff bases with drugs containing amine groups. This bond is highly unstable and can be stabilised by reduction with sodium borohydride. The coupling and subsequent reduction can be achieved in one step. Unfortunately, this reaction may also result in the formation of hemiacetals that reduce the degree of oxidation along the chain and potentially affect coupling reactions



Scheme 2.



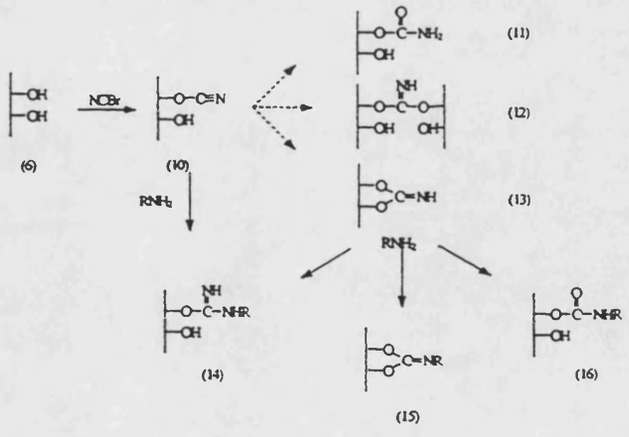
Scheme 4.

[85]. Chemical modification of this type also greatly effects the structure of the carrier backbone which will affect its physiochemical properties and potentially its toxicity and immunogenicity [5,83].

Another attractive method is the use of cyanogen bromide. This leads to less alteration in the basic structure of the carrier but does result in the introduction of positive charges into the molecule (Scheme 3). The reaction is not well understood but it is thought that, along with the desired electrophile, three other preliminary products are produced. The imidocarbonate structures are commonly produced in activation of dextran, particularly the stable five-membered ring structure (13). This can then form the desired linkage when treated with an amine but can also form other linkages which can result in a charged dextran [85]. Thus, this approach, although the chemistry is easy to perform, is of limited use as reliable and reproducible ligand-carrier structures are not formed [5].

The final method of activation is through the use of carbamate esters. Treatment of dextran with 4-nitrophenyl chloroformate results in a highly active carbamate which couples easily with amine-containing drugs [83]. Again, it is thought that some inter-chain cross-links are formed due to the close vicinity of the hydroxyl groups (Scheme 4).

Dextran has a renal threshold of approximately 55000 Da and fractions of higher molecular weights undergo slow partial depolymerisation



Scheme 3.

due to the action of dextranases. Studies with dextran T-40 demonstrate a rapid clearance from the bloodstream; this is thought to be due to three excretory mechanisms. The main fraction which is below the glomerular filtration threshold is excreted in the urine. A small amount passes into the gastro-intestinal tract where it is degraded in the colon. Another small fraction passes across the endothelium and into the interstitium where it is recycled via the lymphatic system. The remaining fractions are taken up over time into cells of the reticulo-endothelial system, such as macrophages and Kupffer cells of the liver, where the polysaccharide is slowly degraded [85]. The distribution of dextran in vivo can be greatly changed by chemical modification. A cationic derivative, diethylaminoethyl dextran, can be formed by treatment of dextran with diethylaminoethyl chloride and an anionic form, carboxymethyl dextran, by treatment with chloroacetic acid (Fig. 6) [82,84].

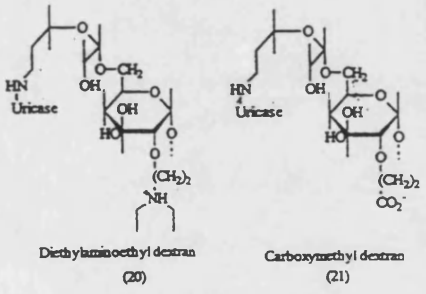


Fig. 6.

Equally, incorporation of a spacer unit for attachment of a drug molecule can result in the formation of cationic and anionic derivatives of dextran [82]. The conjugation of mitomycin C (MMC) to cyanogen bromide-activated dextran via a 6-aminohexanoyl spacer results in a positively charged dextran molecule, whereas spacers derived from 6-bromohexanoic acid give negatively charged polymers (Fig. 7).

Studies on both these types of charged polymers reveal altered distributions in vivo. A cationic conjugate with uricase was rapidly cleared from plasma whereas an anionic derivative has an increased half-life. Measurements of hepatic uptake and urinary excretion gave similar results. Uricase was taken up faster when conjugated to neutral dextran [85]. This rate was enhanced by conjugation to cationic dextran and was reduced by conjugation to anionic dextran [84]. With the mitomycin C derivatives, studies were undertaken in tumour-bearing mice. Here the cationic derivative was rapidly cleared, whereas the anionic derivative had an increased blood residence time, resulting in accumulation in the tumour. These results suggest that intentional incorporation of anions into a dextran macromolecular pro-drug would result in an enhanced delivery system. An alternative approach to the modification of distribution in vivo is the use of lectins to target a polymer to particular cell lines. In early studies, Vansteenkiste [34] investigated the effect of pendant D-galactosylated dextrans on distribution. Plasma clearance was higher than for non-derivatised dextran and hepatic uptake was increased. An interesting finding from this study was that dextrans carrying fluorescein-thiocarbamate alone also exhibited an increased disposition in liver. Thus, it can be assumed that the changed distribution in vivo owing to changes in the carrier could be augmented or overridden by attachment of drug.

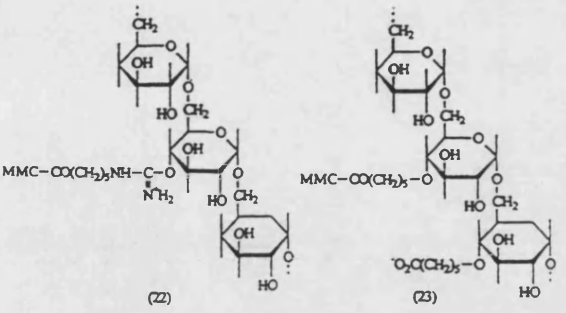


Fig. 7.

Dextran is degraded in vivo by the action of dextranases. These enzymes are absent from blood but are present in the liver, kidney, spleen and parts of the gastro-intestinal tract. Dextranases can be either *exo* or *endo* in their action. Dextran is only degraded very slowly by these enzymes and high molecular weight dextrans can

be captured by the reticulo-endothelial system before they are degraded. Thus, it is important to consider degradation of both dextran and dextran derivatives by isolated dextranases and tritosomes. In initial studies, Schacht et al. [83] investigated the degradation of dextran derivatives in vitro by an *endo*-dextranase. Three derivatives were assessed, a reduced dextran dialdehyde, formed from periodate activation and reduction, an amine derivative, formed by the reaction of 2-hydroxypropylamine with chloroformate-activated dextran, and a succinate derivative. Degradation was diminished by periodate activation and this reduction was proportional to the degree of activation. The other derivatives gave similar results, suggesting that enzyme activity is affected by the degree of derivatisation, rather than the type of modification. The degradation of these macromolecules in tritosomes was also assessed. Dextran itself was slowly degraded and both the urethane and succinylated dextrans showed even lower degradability. Interestingly, the reduced dialdehyde had, in this case, a higher rate of degradation. This was considered to be a combined effect of both hydrolytic and lysosomal degradation. These workers suggested that the degradation of dextran in lysosomes is achieved by dextranases with both *endo* and *exo* characteristics. The same workers have also investigated the lysosomal degradation of dextran, cationic dextran dialdehyde and anionic dextran monosuccinate ester [86]. Results were obtained for both the degradation index and degree of glucose release, i.e., the extent of *exo*-dextranase activity. Liberation of glucose was the same for both dextran and the derivatives. However, the degradation rates of the derivatives were lower. This confirms that dextran is degraded in lysosomes by more than one enzyme. It was proposed that the enzyme with *exo* activity was α-glucosidase and the *endo* enzyme was 1,6-α-D-glucose-6-glucanohydrolase.

Crepon et al. [87] have examined the degradation of dextran derivatives that bear more resemblance to macromolecular pro-drugs. Carboxylic acid, benzamide, and benzylamine-sulphonated derivatives of dextran T-40 were prepared and degraded using an *endo*-dextranase (Fig. 8). Incorporation of increasing numbers of carboxylic acid groups resulted in a linear reduction in degradation. Comparison of polymers derivatised with carboxylic acid groups and polymers derivatised with both carboxylic acids and benzylamine-sulphonated groups showed that the benzylamine-sulphonated groups had a greater inhibitory effect on lability to dextranase. Interestingly, some dextranase action still occurred, even at 80% substitution.

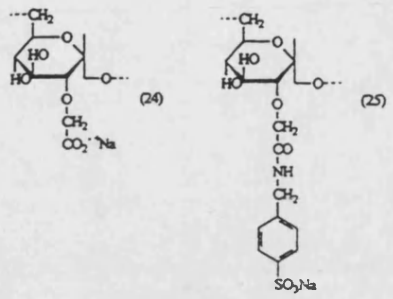


Fig. 8.

The effect of larger substituents has also been investigated [88]. Dextrans have been prepared bearing pendant poly(ethylene glycol) chains, which were degraded both with dextranase and tritosomes. All the derivatives were degraded by dextranase but the extent of degradation was substantially reduced with longer poly(ethylene glycol) chains. With the lysosomal enzymes, no degradation of the derivatives was seen in 36 h. These results suggest that dextran is suitable as a carrier molecule, as it is not degraded in the blood stream, where there are no dextranases, but is very slowly degraded in lysosomes. To afford efficient release of drug from dextran, it may be appropriate to use lysosomally degradable spacers.

The immunogenicity of dextran has been reviewed by Rihova and Riha [89]. Immunogenicity is dependent on the molecular weight of the dextran. Dextrans with molecular mass 51000 Da have very low immunogenicity whereas injection of dextran with molecular mass >90000 Da results in the formation of antibodies during 3 weeks. It has also been shown that the immune effect depends on the dose injected; for high molecular weight dextrans, an immune response is induced at low doses but tolerance is seen at higher doses. The immunogenicity of dextrans is also structure-dependent, branched polysac-

charides being more immunogenic. Two main specificities of antibody are raised to dextran, one with a combining site for isomaltotriose and one with a larger combining site for isomaltohexose. The immunogenicity of modified dextrans must also be evaluated, as this provides information on the macromolecular pro-drug situation. Crepon [87] evaluated the antibody response to a dextran T-40 derivative with 54% carboxylic acid groups and 19.5% benzylaminesulphonated units. The polymer was injected three times over 14 days, with or without complete Freunds adjuvant, into Balb/c mice. The antibody titre was low, even in mice treated with the adjuvant, and the conjugate can be considered to have low immunogenicity. Despite the low immunogenicity of dextran, occasional anaphylactic reactions are seen but the development of a pre-immunisation technique has reduced the risk to 1 in 85000 [5].

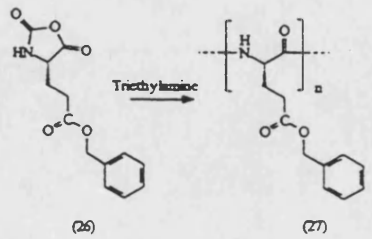
Sezaki et al. have developed pro-drugs of mitomycin C based on dextran [90]. These workers employ hydrolysable spacer bonds, rather than lysosomally degradable bonds, in order to achieve release of the acid-unstable drug both within the cell and within the tumour mass. This could, however, result in the release of the drug in other regions of the body, with consequent toxicity. Mitomycin C has been attached to cyanogen bromide-activated dextran thorough a variety of bonds [5,82,84]. In a recent study [90], three spacers of varying length were evaluated with respect to rate of release of drug and effectiveness in vivo. Drug release rates in vitro demonstrated an increased half-life with increasing chain length but, in cell culture experiments, growth inhibition increased as the length of the spacer decreased. Thus, these two effects must be balanced to provide the most selectively active conjugate. Studies in vivo have shown that a 6-aminohexanoyl spacer provides the largest increase in life-span combined with the largest therapeutic index.

5.4.2. Inulin

Inulin is a vegetable-derived polysaccharide based on fructose units, which is terminated at one end with a sucrose unit. It has limited solubility and is degraded by acid. It is a small polysaccharide, with molecular mass <10000 Da, and is used clinically for testing renal filtration rates, as it is excreted rapidly without degradation in a normal kidney. Activation of inulin using the standard methods mentioned above proved unsuccessful. Periodate activation resulted in only 50% active units, owing to the high level of formation of hemiacetals. An alternative activation method has been developed which is also suitable for the activation of dextran [91]. Treatment with epichlorohydrin yields a 3-chloro-2-hydroxypropyl derivative which can react easily with amine drugs. Succinylated derivatives can also be prepared which can then be activated with 1,1′-carbonyldiimidazole [92,93]. Inulin has been used to a limited extent in preparing macromolecular pro-drugs, mainly as a carrier for the anti-arrhythmic drug, procainamide [85].

5.5. Poly(amino acids)

Poly(amino acids) can be considered to be synthetic proteins. The use of these macromolecules as carriers has been explored for many years. The degradability of poly(amino acids) is a highly attractive feature as is the ease of chemical modification. However, the use of many macromolecules of this type is restricted due to their general cell toxicity. Polymers of most amino acids have been prepared and evaluated. The most popular systems are poly(glutamic acid), poly(lysine), poly(hydroxyethylglutamine) and poly(hydroxyethylaspartamide). Poly(glutamic acid) (poly(Glu)) can be easily prepared by base-initiated polymerisation of the *N*-carboxyanhydride of L-glutamic acid γ-benzyl ester (Scheme 5). Deprotection reveals the desired polymer. The polymerisation reaction can be controlled to produce polymers of a wide molecular weight range.



Scheme 5.

Although attachment of amine-containing drugs to the carboxylic acid would appear to be easily achievable using standard peptide chemistry, some workers have found difficulties with activation procedures. Activation with carbodiimides can result in the formation of cyclic imides, leading to chain scission, or to inactive *N*-acylisoureas. These problems can be overcome by using 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) to form a mixed anhydride (Fig. 9) [94,95].

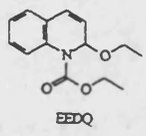
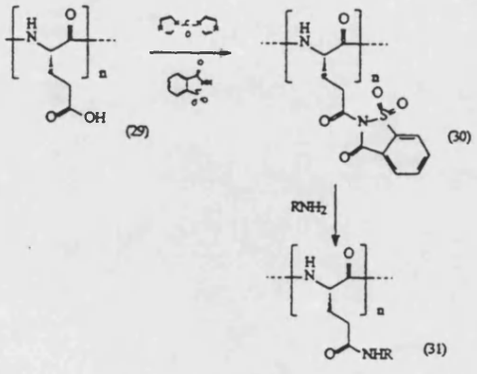


Fig. 9.

The same workers have also developed an alternative coupling method for the attachment of peptide spacers to the macromolecule [75]. Poly(Glu) can be activated using a CDI-saccharin method and then coupled with tetramethylguanidinium salts of oligopeptides to produce the spacer [75,95] (Scheme 6). A stepwise incorporation of amino acids is also possible using a modification of this method in which the CDI can be regenerated using phenyl dichlorophosphate. With both methods, the drug can then be coupled to the polymer via the EEDQ method [94,95].



Scheme 6.

Complexes of poly(Glu) can also be prepared. The polymer has an inherent negative charge and a non-covalent complex of this and *cis*-dichlorodiammineplatinum(II) has been made and evaluated as a pro-drug [96]. Hoes et al. [94] have investigated poly(Glu) macromolecular pro-drugs of adriamycin with respect to their activity in vivo and in vitro, rate of release of drug and immunogenicity. In studies involving papain in vitro, release of adriamycin was achieved only when a peptide spacer was incorporated between poly(Glu) and the drug molecule. This is to be expected, as papain has specificity for the cleavage of α-amide bonds. Further studies evaluating the effect of spacer concentration gave surprising results. Cleavage of the spacer is expected to be faster in polymers with a few spacer units, as enzyme access is facilitated. However, with these conjugates, degradation proceeded faster with polymers bearing many chains. These authors considered these results to be due to degradation of the peptide backbone by the enzyme. Later studies using lysosomal enzymes in tritosomes confirmed many of these results. Release was obtained slowly in the direct conjugate, owing to degradation of the polymer backbone. Release from a conjugate with a GlyGlyGlyLeu spacer was faster, indicating faster degradation of the spacer unit than the polymer backbone [96].

Poly(Glu) is unlikely to be immunogenic. Many workers have shown that the degree of immune response is dependent on the number of different amino acids in the molecule; thus, polymers of four amino acids are considerably more immunogenic than homopolymers [89]. In studies of poly(Glu) in rabbit, guinea pig and man, no antibodies were raised to the polymer. These results were confirmed by studies of an adriamycin conjugate of poly(Glu) in mice. Antibodies were raised both to a direct conjugate of drug to polymer and to a conjugate incorporating GlyGlyGlyLeu but at lower levels than the response to bovine γ-globulin [75]. It would be interesting to develop these studies further. Both adriamycin and spacer units have been shown previously to act as haptens. Thus, the antibodies raised should be examined for cross-reactivity to determine whether their combining site is part of

the polymer backbone. The cytotoxicity in vitro and in vivo of these conjugates has also been evaluated [75,94]. Initial experiments, using both directly-bound and spacer-bound adriamycin, suggested that the compounds were completely devoid of activity against L1210 and B16 cell lines. However, carriers with a biodegradable spacer gave increased survival times, when assessed in vivo in mice inoculated with B16 melanoma. Not surprisingly, the direct conjugate was also inactive in vivo.

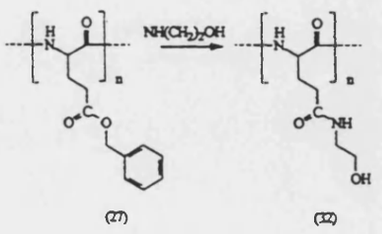
Poly(L-lysine) would appear to be an ideal macromolecular carrier from many viewpoints. It is available in a wide range of molecular weights, has a high number of amine groups for attachment of drug molecules, is biodegradable and can stimulate endocytosis due to the interaction of positive charges with the negatively charged cell membrane. Poly(D-lysine) has all these advantages except that it is not biodegradable, owing to its stereochemistry [97]. Poly(L-lysine) was used frequently in the early years of macromolecular pro-drug development. It was used mainly as a carrier for methotrexate. In these studies, the intracellular mechanism of pro-drug action was confirmed, as conjugates were active in cells without transport mechanisms for methotrexate [8,52]. Poly(D-lysine) was also an excellent tool in the development of lysosomally degraded spacer arms. Release of a drug from a poly(D-lysine) conjugate with a spacer can only occur if the spacer is degraded [8]. More recently, however, the use of both these carriers has declined owing to their general cellular cytotoxicity, a result of their potent non-specific interaction with biological membranes. Poly(L-lysine) is known to have antibacterial, antiviral and antitumour activity. These properties are thought to be due to interactions with the cellular membrane or parasite coat. Unfortunately, the charged interaction that provides these positive effects can also lead to toxicity as it is not tumour-cell specific. In a comprehensive study, Choksakulnimitr et al. [98] investigated the effect of numerous macromolecules on cultured bovine brain endothelial cells, a model of the blood-brain barrier, mouse peritoneal macrophages, a model of Kupffer cells, and rat hepatocytes. Damage to the cell membrane was measured by the release of lactate dehydrogenase (LDH). With Poly(Lys) of high molecular mass (39880 Da), severe leakage of LDH was observed in all the cell lines, while with a smaller polymer (8000 Da) release was reduced, especially in macrophages. It has been suggested that the flexibility of poly(Lys) plays a major part in the release, as many interactions can be made on the cell surface leading to membrane disruption. These results are particularly worrying, as polycations have been shown to accumulate in the liver in vivo and suggest that the toxicity of poly(Lys) may be too high for use in macromolecular pro-drugs.

The immunogenicity of poly(Lys) is more debatable. In some animals, antibodies can be raised to the homopolymer but the response in a particular species is unpredictable [89]. Furthermore, initial studies by Maurer suggested that the polymer was non-immunogenic in rabbits but it has now been shown that, at low levels, immune responses do occur [99]. In a recent study, the immunogenicity of poly(D-Lys) has been investigated in rabbits. Antibodies of both IgM and IgG were raised in response to an initial inoculation with the polymer. On a further administration, a booster effect was seen consisting only of IgG antibodies. The antibodies were isolated and were only reactive against poly(D-Lys). No activity was seen with monomers, suggesting that at least part of the antibody determinant is the peptide bond, or with poly(L-Lys), showing stereochemical specificity. These results suggest that both poly(L-Lys) and poly(D-Lys) are slightly immunogenic. This does not preclude their use in macromolecular pro-drugs but it should be noted that the immunogenicity is likely to be enhanced by inclusion of spacers or drug molecules.

The concerns about the toxicity of poly(Lys) have led to the development of new approaches to using the polymer as a drug carrier. Monsigny et al. have investigated a conjugate of poly(L-Lys) with MDP and sugar targeting moieties in which any free lysine ε-amines have been acylated with δ-gluconolactone. This considerably reduces the adverse properties of the polymer whilst retaining the positive properties. Thus, the polymer is still degradable but is

electrically neutral and has a higher water-solubility [100,101]. Immunogenicity studies have shown that antibodies are not raised to this conjugate [37] and the neutralisation of charge results in a decrease of non-specific cell binding and toxicity. Hudecz et al. have investigated branched polypeptides based on poly(Lys) as carriers [102]. Branched polypeptides have been used as synthetic antigens as the antigenicity of amino acid polymers is increased in polymers with many different components [89]. Thus, before branched polypeptides can be used as carriers, non-immunogenic structures must be found. In a large study of the toxicity of these carriers, it was found that inclusion of other amino acids onto a poly(L-Lys) backbone can reduce the toxicity to cell cultures, both rat liver and mouse spleen cells, whilst the positive cytotoxic effect against cervical cancer cells is maintained. Hence, although poly(Lys) is not a suitable candidate for a drug carrier owing to its high level of non-specific toxicity, modifications of the structure could lead to suitably non-toxic molecules.

Poly(hydroxyethylglutamate) (PHEG) (32) is a water-soluble, neutral, biodegradable and biocompatible polymer that has been proposed as a plasma expander. It is easily prepared and can be reliably activated, making it a suitable candidate as a drug carrier. PHEG can be synthesised from poly(γ-benzylglutamate) via an aminolysis reaction with 2-aminoethanol (Scheme 7) [103]. Treatment with 2-aminoethanol alone can result in chain scission due to aminolysis reactions on the backbone amides. This problem can be avoided by the use of 2-hydroxypyridine as a catalyst. With a 5-fold excess of the catalyst, complete conversion was achieved with minimal chain loss [104].



Scheme 7.

The single hydroxyl group can be easily activated by treatment with 4-nitrophenyl chloroformate. In contrast to dextran, the desired carbonate ester is not rearranged and inter- and intra-chain cross-links do not occur. A useful property of this reaction is that the degree of activation is proportional to the concentration of chloroformate and thus can be tailored for particular requirements [103]. Quantitative couplings with the amino groups of both targeting moieties, such as sugars, and drugs, such as melphalan, can be achieved within 24 h. PHEG is known to be biodegradable. However, in an extensive study, Pytela et al. [105] have investigated the role of particular enzymes in this degradation. PHEG was incubated with both thiol proteases, such as papain and cathepsin B, which have a lysosomal type activity, and serine proteases, such as chymotrypsin and elastase, which are found in blood. Interestingly, the homopolymer was resistant to degradation by the serine proteases and was degraded by the thiol proteases, suggesting a potential lysomotrophic carrier. Unfortunately, when the same workers investigated the effect of inclusion of a hydrophobic group on the side chain, they found that the conjugate was susceptible to serine proteases. This could mean that the selectivity of degradation could be abolished by attachment of a drug. Not surprisingly, the degradation of PHEG was reduced by incorporation of some of the D-amino acid and abolished in D-PHEG. PHEG has been investigated mainly as a carrier of mitomycin C. Studies in vitro have demonstrated the potential of peptide spacers to afford release of the drug within lysosomes whilst retaining hydrolytic stability in the blood stream but the conjugates have not been investigated further [76]. Another approach in the use of PHEG is to co-polymerise it with other amino acids to produce macromolecules with different properties and biodistribution [105,106].

Poly(α,β-*N*-(2-hydroxyethyl)-DL-aspartamide) (PHEA) has also been evaluated as a plasma expander and a drug carrier, although it has not been widely used at present. PHEA has a similar chemical reactivity to PHEG, thus it can be activated to the nitrophenyl carbamate and coupled to amine-containing molecules such as

sugars [40]. Coupling of carboxylic acid drug molecules can also be achieved by the use of carbodiimide reagents [107]. For other drugs such as acyclovir, with a hydroxyl group, the drug molecule can be pre-activated by treatment with succinic anhydride. A carbodiimide coupling can then be performed between the carboxylic acid of the drug derivative and the hydroxyl of the polymer. Interestingly, this approach also results in the inclusion of a spacer unit [108].

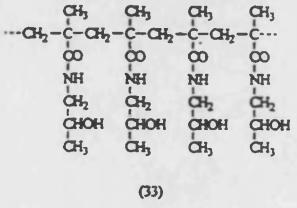
5.6. Hydroxypropylmethacrylamide (HPMA)

Hydroxypropylmethacrylamide (HPMA) (33) and its co-polymers have been investigated as blood plasma expanders and drug carriers. HPMA is a synthetic vinyl polymer produced by radical precipitation polymerisation (Fig. 10) [109]. The homopolymer is non-immunogenic [89] and is easily activated to allow the attachment of drug moieties, making it an almost ideal macromolecule. One potential limitation of the system is its lack of biodegradability, which restricts the use to macromolecules with a molecular weight of less than the glomerular filtration threshold.

The homopolymer is itself inert. However, the hydroxyl groups can be activated to allow attachment of drugs and enzymes. One of the easiest methods of activation is to treat HPMA with cyanuric chloride (Scheme 8) [110]. The resulting active species reacts easily with enzymes in aqueous solution.

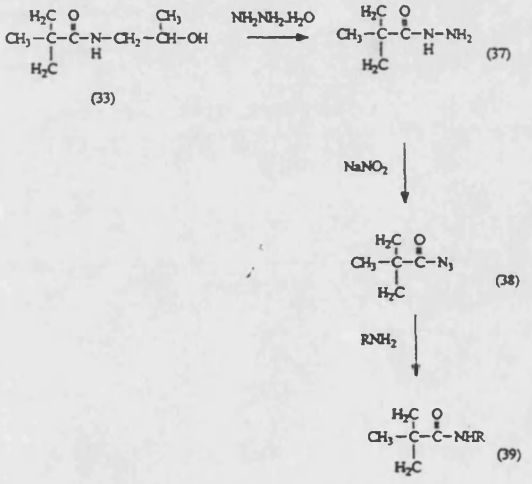
Hydrazide derivatives can also be prepared, which when activated as the azide, can be coupled to amine-containing drugs (Scheme 9).

Activation of the polymer can also be achieved by the use of cyanogen bromide [33]. Although

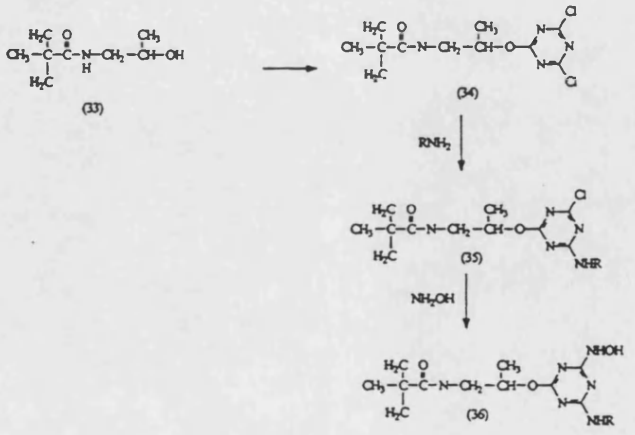


(33)

Fig. 10.



Scheme 9.



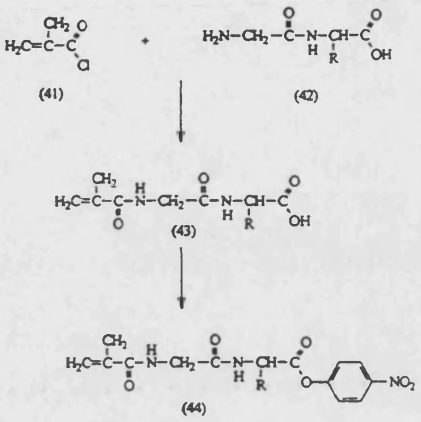
Scheme 8.

this reaction is versatile and can be tailored for the degree of reactivity required, it can result in inter- and intra-chain reactions. All these methods do result in substantial changes in the structure and charge of the homopolymer that may affect both its distribution and immunogenicity in vivo. An alternative approach is to use an active ester [111]. Treatment of the homopolymer with 4-nitrophenyl chloroformate provides an active polymer [112]. Aminolysis reactions with amino acids or drugs results in the restoration of the original polymer structure [113]. This is the most useful and versatile method of activation available (Scheme 10).

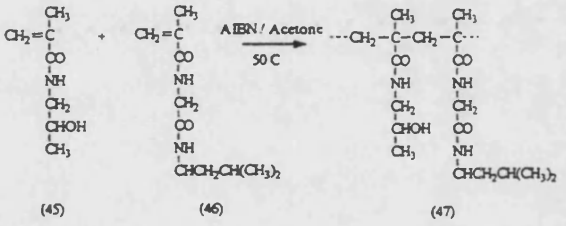
Activation of the homopolymer is, however, not suitable for reproducible levels of incorporation of drug. The alternative is to use co-polymerisation method of HPMA with derivatised methacrylamide units which can easily be achieved. This allows a more accurate and reproducible interaction of reactive groups into the polymer whilst maintaining the majority of characteristics of the homopolymer. Kopecek et al. [109] have developed this approach and have used co-polymers of HPMA incorporating peptide spacers for delivery of many drugs. The general method of synthesis involves *N*-methacrylylation of an amino acid or dipeptide units under Schotten-Baumann conditions. The α-amino carboxylic acid can then be activated to a nitrophenyl ester (Scheme 11).

Co-polymerisation of these methacrylamide derivatives with HPMA is via a radical method involving an azo initiator such as α,α'-azobis(isobutyronitrile) (AIBN). The co-polymerisation takes place rapidly at 50°C in acetone but the rate of polymerisation and the yield of polymer are reduced in comparison to preparation of the homopolymer. Using this method, up to 10% reactive side chains have been incorporated (Scheme 12).

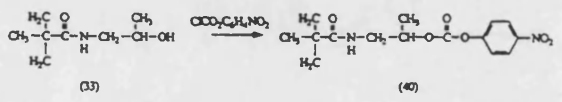
Drug molecules or targeting moieties can be easily incorporated by aminolysis reactions. The reactivity of the nitrophenyl groups on the polymer is considerably reduced in comparison to the monomers owing to steric effects [113]. The rate of reaction is also dependent on the nature of the ester, α-amino esters reacting faster than ω-acylamino esters. With some drugs, it is possible to produce a methacrylylated derivative that can then be co-polymerised. This approach has been utilised by Schacht for the anti-arrhythmic drug procainamide. Co-polymerisation of the derivative with HPMA resulted in a water-soluble drug conjugate (Scheme 13) [114].



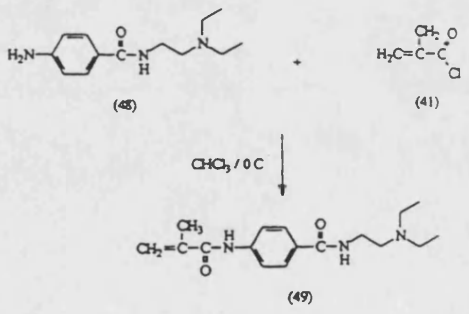
Scheme 11.



Scheme 12.



Scheme 10.



Scheme 13.

Studies with iodine-labelled HPMA provide a value for the glomerular filtration threshold of 45000 Da [11]. Polymers of higher molecular weights remain in the circulation indefinitely as they are non-degradable [41,120]. Intravenous application of a range of HPMA polymers showed clearance from the blood stream to be fairly rapid but dependent on molecular weight. Accumulation in tissue, e.g., spleen and liver, is only apparent with polymers of 778000 Da or above. Thus, most interest has been focussed on polymers of molecular mass <20000 Da to ensure glomerular filtration. As has been discussed earlier, the distribution of HPMA co-polymers in vivo can be altered easily by incorporation of non-specific hydrophobic groups and charge or by the use of specific targeting residues. In tumour-bearing animals, conjugation of drugs to HPMA co-polymers results in an increased concentration of the drug in tumours, suggesting that the co-polymer is tumour tropic [70,71].

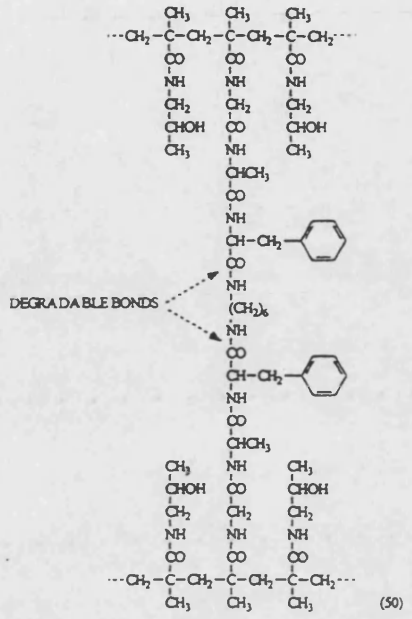


Fig. 11.

Co-polymers of HPMA cannot be degraded by enzymes in the blood stream or lysosome. This could lead to an unacceptable accumulation of the polymer within the body. Hence, with these linear polymers, the molecular weight must be kept below the renal threshold, potentially reducing the tumour tropic effect. Accumulation of the polymers can be avoided by the use of soluble cross-linked polymers. By careful choice of the cross-link, spacers can be prepared which only degrade within the lysosome, i.e., after delivery of the drug. The resulting low molecular weight polymers can be excreted in the urine. An example of a cross-linked HPMA co-polymer is shown in Fig. 11 [116]. The cross-linking reaction is designed to occur below the gel point in order to produce a water-soluble rather than an insoluble polymer. This reaction has to take place at high dilution owing to the low solubility of the reactants. However, this could result in the formation of cyclised structures with the amine reacting twice with the same polymer chain. This problem can be avoided by cross-linking the polymer through short diamines, such as ethane-1,2-diamine. This, however, could lead to decreased enzymatic degradability. Cyclisation can be more easily overcome by employing a two-step reaction. Treatment of the polymer with a large excess of the diamine will minimise cyclisation and maximise the formation of free amines. In a second step, the free amino groups can then be allowed to react with more polymer starting material [109]. Two-stage cross-linking also allows the formation of very high molecular weight products. Initially, model studies on the degradation of peptide sequences between the bis-amine and polymer were performed. Amino acid sequences were prepared which showed similar specificity as side-chains for the enzymes chymotrypsin [117], trypsin 118] and papain [119]. In all cases, degradation was reduced in comparison to the same chains attached to the linear polymers. This could be due to either decreased access of the enzyme to the bond to be cleaved or to conformational constraints of the amino acid sequence as it is tethered at both ends.

This decrease in degradability was especially pronounced with cross-linked polymers with either a two amino acid peptide sequence or a short diamine [117,119]. Cross-linked polymers have been evaluated in vitro for lysosomal degradation using isolated cathepsin B. Degradation was again highest with long amino acid chains or with a long α,ω-diamine [57]. In a further study, cross-linked polymers with the amino acid se-

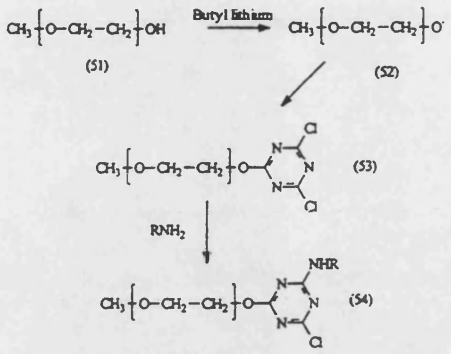
quence GlyGlyPheTyr were degraded easily by rat lysosomal enzymes but were resistant to degradation in plasma. Preliminary experiments with the same polymer in vivo in rats confirmed that degradation takes place, as low molecular weight chains were excreted [120]. This approach has not been investigated further in the development of intravenous agents for the treatment of cancer, although HPMA cross linked polymers formed above the gel point are being evaluated for colon specific drug release [121].

The immune response to both HPMA and co-polymers of HPMA has been thoroughly investigated by Rihova [89]. Injection of the homopolymer does not appear to result in an immune response in mice as no antibody titre is measurable. Incorporation of peptidyl side chains, however, does result in the production of antibodies. This is a weak reaction, the titre is considerably lower than for a model immunogen, bovine gamma globulin, and the antibodies are of the weak IgM class [122]. Injection of a wide range of doses of the co-polymers demonstrated a tolerance reaction; the antibody response to most co-polymers was higher for a 10 $\mu g\ kg^{-1}$ dose than for a 100 $\mu g\ kg^{-1}$ dose. The structure of the peptide also had an effect on the antibody titre but no structure-activity relationships could be evaluated. Interestingly, using one peptide spacer at different substitution levels did not affect the number of antibodies raised. This suggests that the presence of only one or two epitopes, as in the 1 mol% conjugate, can elicit an antibody response [122]. The specificity of the antibodies has also been investigated in cross-reactivity reactions. These showed that the majority of antibodies formed were directed to the peptide sequence and therefore could not cross-react but some were raised against the polymer backbone, most probably against the hydroxypropyl unit. This suggests that, although antibodies are not raised against the homopolymer, once the entire molecule becomes immunogenic due to the peptide chain, antibodies are raised against all parts of the molecule. Similar results were obtained with co-polymers bearing adriamycin. A low antibody response were seen at all doses and was unaffected by the incorporation of galactosamine targeting residues [115]. The effect of incorporation of known haptens has also been investigated. Again, the majority of antibodies were raised against the hapten. With this system, the effect of molecular weight on immunogenicity was evaluated. More antibodies were raised to the hapten with a co-polymer of molecular mass 200000 Da than for one of 5000 Da [89]. The effect of pre-immunisation on the efficacy of a daunomycin HPMA co-polymer has also been evaluated. Pre-immunisation can either give no effect, an adverse toxic affect or result in loss of activity of a drug. In this system, pre-immunisation over 35 days prior to inoculation of the mice with L1210 cells resulted in no loss of activity of the conjugate [123].

5.7. Poly(ethylene glycol) (PEG)

Poly(ethylene glycol) is a non-toxic, non-immunogenic, highly water-soluble linear polymer [124]. It has mainly been used medically in the modification of therapeutic proteins. Modification of proteins with PEG leads to a decrease in the toxicity and immunogenicity of the protein, whilst increasing both the water solubility and plasma half-life [124–126]. These properties suggest that conjugation of a drug to PEG or PEG co-polymers would provide an ideal non-toxic macromolecular pro-drug with enhanced plasma lifetime and potentially increased tumour uptake. Much research has been directed towards the preparation of activated PEG for attachment to proteins. The coupling reaction in this system is usually between PEG monomethyl ether and the ε-amino group of protein lysine residues [124]. Therefore, most active derivatives are based on carboxylic acids. For the conjugation of drug or the formation of block co-polymers, amine residues may be required. These can be provided by treatment of carboxyl-activated PEG with an α,ω-diamine or by direct conversion of PEG to a PEG-amine [127,128].

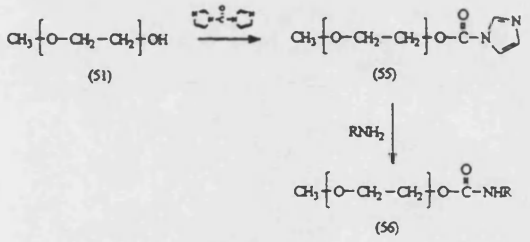
Activation of PEG with cyanuric chloride is one of the oldest and most widely used methods for protein coupling. The original method of Abuchowski and Davis has been modified by Harris [129] to enable complete conversion within 2 h, through the use of alkoxides (Scheme 14). Coupling of proteins using this method has been



Scheme 14.

linked to inactivation of the protein [125,130], cross-linking and non-specific coupling to OH and SH residues. Thus, this method has been overtaken by more recent advances.

Activation using carbodiimides (Scheme 15) is effective and does not lead to cross-linking. However, the coupling reaction is slow and can take up to 72 h. This method, therefore, is of little use in co-polymerisation reactions.



Scheme 15.

Treatment of PEG with succinic anhydride generates a carboxylic acid which can be activated via a DCC coupling to form the *N*-hydroxysuccinimide ester (Scheme 16) [130]. These derivatives react quickly with amines [132] but the resulting product can be unstable owing to hydrolysis of the ester linkage.

An alternative approach, developed by Zalipsky, allows the formation of a *N*-hydroxysuccinimide-activated PEG without hydrolytic instability. Treatment of PEG with phosgene followed by *N*-hydroxysuccinimide results in a stable urethane derivative between an amine drug and PEG (Fig. 12) [132].

These are only the main methods used. Activation and coupling have also been achieved by reductive amination with PEG aldehyde, by epoxide formation with epichlorohydrin [133,134], and formation of tresylates (2,2,2-trifluoroethanesulphonates).

A number of co-polymers of PEG have been prepared for use in drug delivery. Zalipsky [132,135] utilised the *N*-hydroxysuccinimide method to prepare a co-polymer of PEG and lysine with the amino acid linked through the α- and ε-amino groups (Scheme 17). The degree of polymerisation could be easily altered by varying

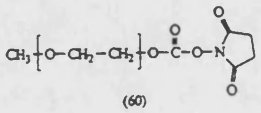
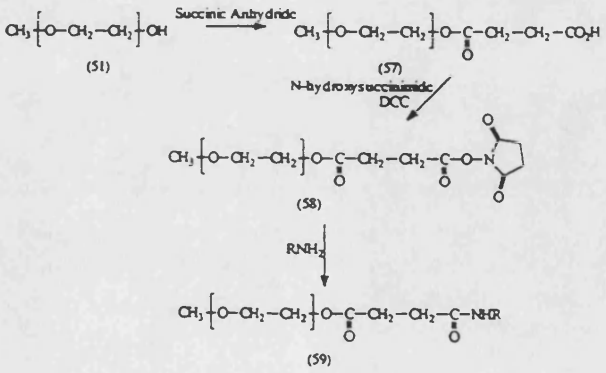
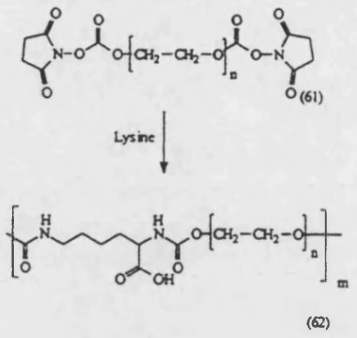


Fig. 12.



Scheme 16.



Scheme 17.

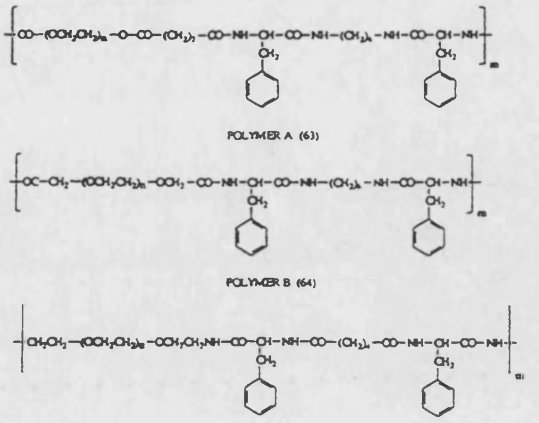


Fig. 13.

the reaction time and concentration of the reactants. A polymer of molecular mass 170000 Da was achieved in a 2 h polymerisation starting from PEG of mean molecular mass 2000 Da. The co-polymer has pendant carboxylic acids that can be easily activated as azides or active esters [132].

An AB block co-polymer of PEG monomethyl ether with poly(aspartic acid) has also been prepared. In this case, an amine derivative of PEG acts as an initiator for the *N*-carboxyanhydride polymerisation of β-benzyl-protected aspartic acid [133].

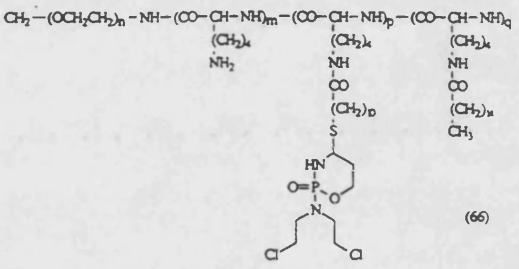
PEG as well, as being chemically inert, is not susceptible to biodegradation. Thus, if high molecular weight co-polymers are to be used for macromolecular pro-drug production, enzymatically degradable units must be introduced to the structure. Obviously, in a co-polymer of PEG and poly(aspartic acid), the aspartic acid unit will be degradable, allowing breakdown of the polymer [133]. Unfortunately, this degradation would not be specific to the lysosomal compartment. However, by developing particular small peptide linkages, it may be possible to produce a selectively degradable co-polymer. Ulbrich et al. [66] investigated the degradability of PEG units linked via diamines incorporating one amino acid, Phe. Three polymers were synthesised, one containing both enzymatically and hydrolytically degradable bonds (polymer A) and two containing enzyme-sensitive bonds (polymers B and C) (Fig. 13). These workers were able to demonstrate the cleavage of all three polymers by chymotrypsin; however, only polymer A was degraded hydrolytically. With polymers B and C, the rate of degradation depended on the orientation of the peptide bond with respect to the PEG chain. In polymer B, more favourable enzyme-substrate interactions are provided, where PEG is in P_2 to P_4 than in polymer C where these positions are occupied by the bis-amine. Thus, the degradation of Polymer B is faster than polymer C. These results demonstrate the ease of producing degradable PEG co-polymers [66]. However, before these polymers can be used as drug carriers, either an amino acid with functionality or a modified bis-amine must be introduced to allow attachment of a drug molecule.

PEG is uncharged and hydrophilic and, therefore, does not interact easily with body components. An example of this is the reduced uptake of microspheres into the reticulo-endothelial system when they are coated with PEG [133]. Studies [134] of the body distribution of PEG injected intravenously to mice show that the half-life is dependent on molecular weight and can vary between minutes and days. Urinary clearance was dependent on molecular mass and a glomerular filtration threshold was calculated to be 30000 Da. Uptake by other organs was unrelated to the molecular weight of the polymer; appreciable accumulation was only seen in the kidney and liver. This accumulation was thought to be due to uptake by macrophages and Kupffer cells.

PEG has low toxicity. Studies in dogs have

shown no toxic or cumulative effects even at the high dose of 90 mg kg^{-1} day^{-1} [124]. Studies in rats have confirmed these results, administration of a 16% w/w oral dose of PEG having no adverse effects. There have been occasional reports of toxicity in man on oral and topical administration. However these reactions are not seen with intravenous application [131]. PEG is also non-immunogenic. Antibodies were not raised to PEG of molecular mass <100000 Da in rabbits, even in the presence of complete Freunds adjuvant. Investigations on the immunogenicity of PEG-protein conjugates have demonstrated a decrease in the antigenicity of the protein. Proteins are highly antigenic due to their heterogeneity. The decrease in immunogenicity, measured by decreased antibody titre, is thought to be due to a shielding effect of the PEG chain, preventing recognition of the haptens [89,124,130]. When antibodies are raised to PEG-protein conjugates, a portion of the antibodies are raised against the PEG chain. Injection of PEG-allergens to humans does result in the formation of anti-PEG antibodies in 50% of the population but these are of the weak IgM type. In fact, 2 years after administration, no circulating antibodies to PEG can be seen. This response, therefore, is not thought to be of clinical significance [124].

The shielding property of PEG may also prove beneficial with PEG block copolymers bearing drugs. Many drugs are very active haptens, so coupling with PEG co-polymers may reduce their immunogenicity. The micellar structures formed by some AB block co-polymers of PEG are also highly unlikely to be immunogenic. Initial studies by Pratten et al. on block co-polymers of PEG and Poly(Lys) substituted with palmitoyl groups gave surprising results [31]. It was expected that pinocytic uptake of this polymer would be high owing to hydrophobic interactions between the palmitoyl groups and the cell surface. However, no improvement over PEG was seen. It was suggested that the block co-polymer forms a micellar structure in which the palmitoyl groups are in a central core and cannot interact with the cell surface. This system was further investigated by Ringsdorf et al., with a drug moiety, an inactive cyclophosphamide, attached to the palmitoyl group. The drug is usually rapidly hydrolysed to an active derivative. However, when attached to the polymer, this hydrolysis was slower. Studies of the cross-linking of DNA due to the drug were evaluated in L1210 cells in vitro. Maximal cross-linking was achieved considerably later with the conjugate than with free drug. It was suggested that the drug was within a micelle core and was only released after cellular uptake [1] (Fig. 14).



(66)

Fig. 14.

More recently, Yokoyama et al. [135,136] have consciously developed a block copolymer prodrug system that spontaneously forms micelles in solution. The system is based on a co-polymer of PEG and poly(Asp), where drug molecules such as adriamycin can be attached to the aspartic carboxylic functions. The co-polymer is easily produced. PEG acts as the initiator for the polymerisation of the aspartic unit and, following deprotection of the polymer, adriamycin can be introduced via an EDC coupling. Incorporation of a drug onto a macromolecule is usually limited by the solubility of the conjugate; as the level of drug increases, precipitation can occur. With these block polymers, however, 50 mol% of drug can be incorporated [136,137]. This property is dependent on the solubilising action of PEG, as similar incorporations cannot be achieved with poly(Asp) [138]. Analysis of these co-polymers gave apparent molecular masses >30000 Da, where the single-chain molecular mass is 9000 Da. This suggests that an aggregation process has occurred, i.e., a micelle has been formed. This is confirmed as these structures are broken on treatment with a surfactant [139]. It has been suggested that single polymer chains exist in an

equilibrium with the micelles and that drug release is only achieved from the single chain [138]. The micellar size can be altered within tight limits by altering the molecular weight of the PEG chain. However, altering either the length of the poly(Asp) chain or the content of incorporated drug does not affect the size of the micelle [138].

These systems have been investigated extensively, both for their distribution in vivo and cytotoxicity [137]. In a distribution study in vivo, the existence of micelles was confirmed. No urinary clearance was measured for two conjugates based on PEG 5000 and 12000 despite the fact that their molecular weights are below the glomerular filtration threshold. The conjugates remained in the blood stream for a prolonged period and were passively accumulated into the tumour. After 24 h, a concentration of 10% of dose per g of tissue was seen for the tumour, whereas for free drug the highest concentration was 0.9%. Coupled with this, the conjugate displays decreased accumulation in the heart, a major organ for toxicity, in comparison to free drug [137,138]. The activity in mice with P388 leukaemia has also been evaluated. The conjugates displayed increased activity and decreased toxicity when compared to the free drug [138]. These workers have also attached an antibody to this system and demonstrated that the micelle structure is lost. In this situation, the single polymer chains form an ideal conformational state in which the disulphide linkage to the antibody is protected by the aspartic acid residues [139–141]. Micelle formation is only possible with block co-polymers of an AB structure. However, it is thought that, in more complicated co-polymers, the molecule will arrange itself into the most suitable conformational state. With PEG co-polymers, the PEG will be exposed to the circulation and hydrophobic units will be protected, as is the case with PEG-substituted proteins [139].

An interesting approach to the use of PEG as a macromolecular carrier has recently been proposed [142]. In this case, one PEG chain is coupled to one drug molecule, e.g., adriamycin, and one targeting group, lactose. This conjugate was active in vitro in both leukaemia and hepatoma cell lines. Despite this efficacy, this system cannot be considered ideal as a large number of conjugates would have to be delivered to the cell rather than one conjugate bearing many drug molecules. Nathan et al. [135] have investigated a co-polymer of PEG and lysine as a carrier for the antibacterial drugs; cephradine and penicillin V. In this approach, the pendant carboxylic acid of the amino acid was successfully converted to other reactive groups, such as amino and hydroxyl, to enable the coupling of a variety of compounds. Both biodegradable and non-biodegradable conjugates were prepared, a number of which also incorporated a spacer.

6. Macromolecular systems for magnetic resonance imaging

6.1. Introduction

The majority of research on macromolecular carriers has involved the investigation of macromolecular pro-drugs for the treatment of cancer. However, almost any drug can be attached to a carrier, to allow targeting or to increase blood circulation time. Macromolecules can also be used in the imaging of tumours and other diseases. A range of macromolecular contrast agents have been produced for use in Magnetic Resonance Imaging (MRI). These are successful not only in the imaging of tumours and inflammation but as blood pool contrast agents to allow investigation of perfusion and occlusion of the vascular system. The basis of contrast enhancement of MR images and contrast agents are considered in this section.

MRI is based on detecting the NMR signals of water protons within the body. Tissues contain varying amounts of water and this is the basis of contrast between organs. This is enhanced by using a gradient magnetic field. The protons then resonate at a different frequency depending on the position within the field. This allows spatial information to be combined with the resonance frequencies and transformed into an image of the organs within the body [143]. When a magnetic field is applied to a proton, the induced magnetism is aligned along the Z-axis. When a radio

frequency pulse is applied this magnetic vector moves away from the axis and then returns or 'relaxes' back to the axis. The time taken for this relaxation is T_1, the spin lattice (longitudinal) relaxation time. A component of the magnetic field lies perpendicular to the Z-axis; this is also perturbed by application of a pulse and relaxes back with a relaxation time T_2, the spin-spin (transverse) relaxation time. So, in MRI, pulses are applied rapidly to prepare images that are based on the different relaxation rates of water protons [143,144].

In some cases, in normal, non-enhanced tissue, the properties of two tissues are not sufficiently different to allow imaging of lesions or tumours. In these cases, a contrast enhancement agent can be used. These can either alter the spin density of a tissue or the relaxation times of the protons within the tissue. Spin density is a measure of the number of protons in a volume. By using gases, such as CO_2, and perfluorocarbons, which have no hydrogen atoms, the total spin density within the area can be reduced, giving a decrease in signal intensity [145,146]. However, most interest has been focussed on altering the T_1 and T_2 times of tissues. Tissues with short T_1 give brighter images than those with long T_1 as the majority of the magnetism is aligned along the Z-axis at a given time. In contrast, tissues with short T_2 values have decreased signal intensity and are seen in images as dark areas. The values of T_1 and T_2 can be altered in vitro and in vivo by the addition of compounds with magnetic properties [143,145].

6.1.1. Diamagnetism

Most molecules are diamagnetic; that is, they have no magnetic dipole as they contain no unpaired electrons. In an applied magnetic field, they do exhibit a small net magnetism which is aligned antiparallel to the applied field. This small local field exerts only a minor effect on the water protons of a tissue. Hence, addition of a diamagnetic ion does not result in enhanced images [147].

6.1.2. Paramagnetism

Paramagnetism occurs when an ion or molecule contains unpaired electrons. Although there is no net magnetism without application of an external field, once an external field is applied orientation of the unpaired electrons results in the formation of a strong local magnetic field. Paramagnetism is prevalent in transition metals, where there is preferential filling of the 4s level giving rise to a number of unpaired electrons in the 3d level, and the lanthanides. Thus Mn^{2+}, with five unpaired electrons, and Gd^{3+}, with seven unpaired electrons, are effective paramagnetic ions [147]. If a paramagnetic ion is introduced into the body, it can interact with the proton nuclei of water molecules to reduce both the T_1 and T_2 relaxation times. The ability of the ion to alter the relaxation times depends upon the ease of approach of the proton to approach the paramagnetic centre and is termed relaxivity. This property depends on the magnetic moment of the ion, which is itself related to the number of unpaired electrons. Thus, Mn^{2+}, Gd^{3+}, and Fe^{3+} ions have high relaxivities owing to their large magnetic moments [145]. The type of interaction can be inner sphere, in which a molecule of water binds directly to the primary co-ordination sphere of the metal ion from which it can exchange with the bulk water. Alternatively, outer sphere interactions can occur, where there is no direct binding. Relaxivity is a combination of these effects, although outer sphere mechanisms are only of interest with chelated ions [140]. Relaxivity is also dependent on the correlation time, which is a term describing the likelihood of an interaction occurring between the unpaired electron and a water molecule. The correlation time is the sum of three correlation times; the rotational T_R, the electron spin relaxation T_S and chemical exchange T_M [143].

$$1/T_C = 1/T_R + 1/T_S + 1/T_M$$

Increasing the rotational correlation time, by slowing molecular motion of a paramagnetic ion, can lead to increased relaxivity. This has been demonstrated by attachment of paramagnetic ions to slow-tumbling protein macromolecules. Also, careful choice of paramagnetic ion can enhance relaxivity; paramagnetic compounds based on Mn and Gd, and nitroxides have slow electron spin relaxation times and high relaxivity,

whereas Dy has a faster electron spin relaxation time and lower relaxivity [145].

Paramagnetics alter both T_1 and T_2. Reduction in T_1 leads to an enhanced signal intensity, whereas reduction in T_2 leads to a loss of image intensity. Thus, a balance must be found between these two effects to achieve contrast enhancement. At low field strengths, the decrease in T_1 dominates the relationship and enhanced images are seen; at higher fields, the T_2 effects dominate and signal enhancement is reduced. The concentration of paramagnetic ions can also alter the imaging effect; at high concentrations T_2 effects dominate. Thus, in producing images, it is important to select the applied magnetic field strength to provide either T_1- or T_2-weighted images. With paramagnetic ions and chelates, the majority of images are produced with positive enhancement T_1-weighted scans.

6.1.3. Ferromagnetism

Ferromagnets are permanent magnets. This is due to a property of a group of atoms or molecules in a solid crystal rather than a single ion. When an external field is applied, the molecules arrange to produce a magnet which remains even when the field is removed. However, if the size of a multi-domain ferromagnet is reduced to less than 350 Å, a single domain particle can be formed. These particles have slightly different properties, although they are rapidly magnetised in an external field like ferromagnets. When the field is removed, they behave as paramagnets and do not retain the induced magnetism. These particles are known as superparamagnets. The most commonly used superparamagnetic contrast agent is based on small particles of magnetite, a naturally occurring iron oxide Fe_3O_4. Superparamagnetics, unlike paramagnetics, have a monophasic action on relaxation times. Thus T_1 relaxation times are barely affected by the administration of a superparamagnet but T_2 is greatly increased, allowing negative enhancement images [147].

Thus, it is possible to design contrast enhancement agents for imaging of disease states by altering the magnetic environment in particular tissues. Initially, free paramagnetic ions were used to produce this effect but these have now been developed further, with the production of metal chelates and the use of pharmaceutical delivery forms, to achieve excellent organ-specific imaging.

6.2. Metal ions

As early as 1948, paramagnetic ions, such as ferric nitrate, were used to enhance the relaxivity of water protons [143]. This phenomenon was exploited in gastro-intestinal imaging but the free ferric ions caused irritation of the gastro-intestinal tract. Further studies using Mn^{2+} enabled the imaging of myocardium in dogs. This cation localises in healthy myocardium but cannot be taken up into infarcted regions. Thus, imaging of infarction was possible. These studies showed that the degree of relaxation enhancement was directly proportional to the concentration of paramagnetic ion in the organ. Although these results were promising, the free paramagnetic Mn^{2+} ion is highly toxic, so cannot be used in MRI. It is taken up by calcium transport mechanisms in the body, resulting in cardiovascular toxicity [145].

6.3. Metal complexes

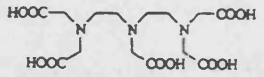
Complexes of paramagnetic ions with co-ordinate ligands have been widely tested as contrast enhancement agents. Although the relaxivity of most of these complexes is reduced in comparison to the free ion, owing to decreased inner-sphere binding of water, the toxicity of the paramagnetic ion is also reduced. Thus, a larger dose can be given, overcoming the diminished relaxivity. The main paramagnetic ions used in these systems are Gd^{3+}, Mn^{2+} and Fe^{3+}, although chromium EDTA complexes were used in initial studies [148–151]. Both cyclic and acyclic ligands have been developed, although at present only one compound, an acyclic compound, is in general clinical use.

6.3.1. Acyclic ligands

The original acyclic ligands were developed from EDTA. EDTA has been used since the early 1950s as a detoxifying agent for iron overload. Since then, it has been shown that modi-

fication of the ligand structure can result in enhanced contrast in a variety of tissues and coupling to targeting moieties can achieve cell line targeting.

The dimeglume salt of Gd-DTPA (Fig. 15) has been used in the clinic for over 10 years in imaging of brain and spinal tumours. Model studies in rats [152] demonstrated the potential of contrast enhancement imaging of radiation damage in the brain. These studies were rapidly advanced into human trials, where cerebral tumours were more accurately contrasted with oedema and normal tissue using the conjugate [153].

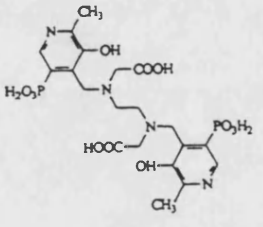


DTPA
(67)

Fig. 15.

Gd-DTPA exerts its effect in an extracellular manner and undergoes rapid renal clearance; it is therefore a useful contrast enhancement for the assessment of renal function. In studies in rats [152], enhancement of the kidney parenchyma was achieved 5 min post injection and was retained for >90 min. The complex has also been used in angiographic studies [154], to determine neurological conditions, vascular occlusion and atherosclerotic disease. Imaging of small vessels not seen without a contrast agent was possible with Gd-DTPA. Despite these promising results, Gd-DTPA is not a universal contrast agent for all disease states. Imaging of liver disease and tumours is not possible, owing to rapid exchange between the normal and diseased tissue, leading to the entire region showing enhanced contrast. Targeting of a DTPA complex has however been achieved, with a radioactive chelate, to malignant melanoma. Two DTPA ^{111}In chelates were conjugated to MSH. This allowed targeting to the α-MSH receptors on malignant cells and gave excellent radiographic imaging. This technology could easily be transferred to the delivery of paramagnetic ions. Phosphonate chelates of Gd have also been prepared to enable targeting to myocardial cells [155].

Gd-DTPA is of low toxicity. A small amount of demetallation does occur but is not significant and the complex is extremely safe in the normal dosage range [145]. However, the charged complex becomes more toxic at higher doses, owing to increased osmolality. More recently, non-ionic complexes derived from DTPA have been prepared. These have comparable molar relaxivities to Gd-DTPA but reduced osmolality and, hence, can be used over a larger dose range. The most commonly used derivative is Gd-DTPA bis-(methylamide) or Gadodiamide injection. As Gd-DTPA cannot be used successfully in the imaging of liver carcinoma, many attempts have been made to prepare similar chelates which are in some way targeted to the liver. Manganese(II) *N,N'*-dipyridoxalethylenediamine-*N,N'*-diacetate-5,5'-bis(phosphate) (Mn-DPDP) (Fig. 16) is designed to be targeted to hepatocytes through the pyridoxal 5'-phosphate moieties. This coenzyme is taken up by a membrane transport system in hepatocytes as is the chelate [156]. It is therefore possible to distinguish between functioning and non-functioning hepatocyte, that is between normal and diseased tissues. Thus, negative contrast enhancement is achieved, the normal cells showing hyperintense relative to the diseased cells. In healthy subjects, enhancement of liver parenchyma was achieved after 1 min and persisted for 30 min. Bile imaging was also possible after 15 min, owing to hepatobiliary excretion of the chelate [157]. Few side effects were seen with this compound, although the majority of subjects did experience facial flushing. Pre-clinical studies in tumour-bearing rats [158] demonstrated a small accumulation of the



DPDP
(68)

Fig. 16.

chelate in the liver and initial images gave no contrast between normal and tumour tissue. After 30 min, however, there is enhanced tumour-to-liver ratio, presumably owing to decreased excretion from the tumour cells. Obviously, the degree of uptake of the chelate by diseased cells depends on the level of normal hepatocyte function retained. Thus, it is possible to achieve differential diagnosis of liver tumours [157,159]. Metastatic liver disease and well-differentiated tumours have no uptake and thus are easily negatively imaged, whereas non-differentiated tumours can only be visualised by a positive enhancement about 24 h after administration.

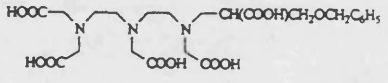
New ligands for gadolinium have also been prepared to allow liver targeting. Gd-BOPTA is a derivative of DTPA with a benzyloxymethyl group in the backbone (Fig. 17). It is intended that this should be taken up by the anionic hepatocyte receptor which takes up bromosulphophthalein [145]. This complex has a high proportion of biliary excretion and is accumulated in liver in rats, where normal cells can be easily imaged [158]. This leads to negative imaging of tumours.

A lipophilic chelate of iron, Fe-EHPG, has been produced (Fig. 18). This is designed to be taken up into rat liver cells through non-specific hydrophobic interactions. Good localisation in the liver and bile was achieved. However, this chelate has lower relaxivity than other chelates and large doses are required; this is a problem as some toxicity has been seen [145]. Studies in mice demonstrated increased signal intensity of both the liver and bile in healthy animals, and enhanced visualisation of intrahepatic implanted tumours and a metastatic model. Increasing the lipophilicity can lead to decreased liver uptake as the chelate binds to serum proteins, therefore the chloro derivative is most widely used owing to its favourable balance of protein binding and liver uptake.

6.3.2. Cyclic ligands

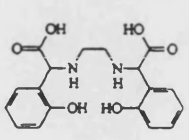
In the search for even more effective chelates, macrocyclic ligands have been produced. Although these complexes take up paramagnetic ions slowly, they exhibit increased stability once the ion is bound and hence reduced risk of toxicity due to leaching. The major ligand that has been evaluated is Gd-DOTA (gadolinium tetraazacyclododecanetetraacetic acid) (Fig. 19). This complex has similar relaxivity to Gd-DTPA and similar biodistribution but is less toxic [158]. It is also has a more enhanced relaxivity at low field strengths, owing to the effect of symmetry on the electron relaxation time.

As with DTPA, non-ionic complexes have been made such as DO3A [145]. Azaphosphonic acid derivatives have also been produced which can be either anionic, cationic or neutral and suitably functionalised for the attachment of targeting moieties. These complexes (Fig. 20) are



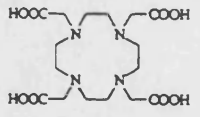
BOPTA
(69)

Fig. 17.



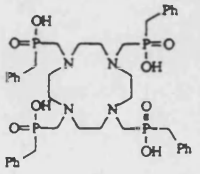
EHPG
(70)

Fig. 18.



DOTA
(73)

Fig. 19.

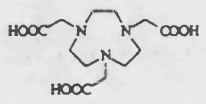


(74)

Fig. 20.

targeted to the liver and bile. Radiolabelled Gd complexes of compound 74 have been shown to clear more slowly from tumour tissue than normal tissue allowing a degree of specificity.

Tumour targeting has also been achieved with indium-labelled NOTA complexes. These compounds accumulate in some skin cancers. However, NOTA is not a good ligand for paramagnetic ions. Paaramagnetic cyclam complexes of Mn(III) and Cu(II) have also been produced (Fig. 21). Mn(III) cyclam is a highly stable complex with comparable relaxivity to Gd-DTPA and localises as efficiently as Fe-EHPG in the liver [144].



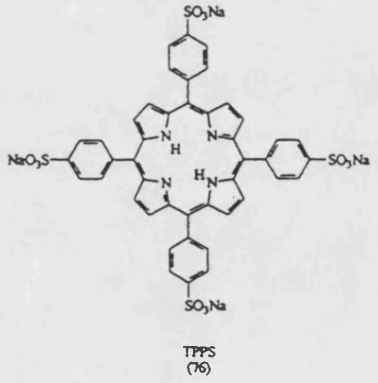
NOTA
(75)

Fig. 21.

Metalloporphyrins are endogenous metal binding ligands. Although they are of complex chemical structure, they are of increasing interest owing to selective uptake by some tumours [146]. Selective uptake of porphyrins into tumour cells was first noted in the 1920s. More recently, this property has been exploited in photodynamic therapy [161]. Some derivatives of haematoporphyrin can be localised to tumours and act as photosensitisers for conversion of triplet oxygen to cytotoxic singlet oxygen. Incorporation of a paramagnetic ion into a porphyrin could, therefore, result in a higher tumour concentration of a contrast agent. Accumulation of metal-bearing polymers in tumour cells has been demonstrated by many workers, although some have suggested that the tumour selectivity of porphyrins can be reduced by metallation. The mechanism by which tumour selectivity occurs remains unknown [162]. It has been suggested that the porphyrin could be trapped in cells by enzymic alteration, or be compartmentalised into specific regions in the cell. Alternatively, it is possible that the porphyrin binds to proteins or fibrous tissue or is taken up into cells by specific transport mechanisms. Studies on the retention of both TPPS and Mn-TPPS in human breast cancer cell lines, confirmed that the most likely mechanism is selective uptake via a porter for TPPS.

Although porphyrins can chelate numerous metal ions, the most useful in terms of imaging is manganese. Gadolinium is too large an ion to form a stable complex so is easily leached and iron loses its paramagnetism over pH 6 when chelated into porphyrins [145]. Manganese fits easily into the porphyrin and these complexes have surprisingly high relaxivities of $>10\ mM^{-1}\ s^{-1}$. Although direct comparisons cannot be made with Mn(III) ions as they are unstable in the circulation, this relaxivity is 3 times higher than that of $MnCl_2$. It has been suggested that the increased relaxivity is due to closer interactions between the paramagnetic ion and the water protons or to symmetry effects [152].

The water-soluble tetraphenyl porphyrin derivative TPPS (Fig. 22) has been most widely evaluated as a paramagnetic chelating ligand. Studies in tumour-bearing mice [163–166] have demonstrated increased uptake of the chelates into lymphomas, fibrosarcomas, colon carcinomas and breast cancer. In all cases, accumulation is seen almost immediately in the tumour; however, the best tumour: normal tissue ratios are achieved after approximately 48 h, when the complex has been excreted from normal tissues. This complex has also been used in imaging brain glioma [167]. Unlike Gd-DTPA which perfuses out of the tumour, the porphyrin appears to bind tightly to the tumour cells, resulting in enhancement of the tumour image for >4 days. The



TPPS
(76)

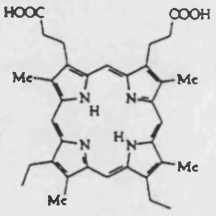
Fig. 22.

porphyrin complex is also highly efficient in distinguishing tumour from normal tissue and oedema. In this situation, it has been proposed that the selective uptake occurs through peripheral benzodiazepine receptors which are abundant in tumour cells but not in normal cells. The versatility of porphyrins suggests that there is potential, through chemical modification, of achieving selective uptake into almost any organ or tumour.

Although Mn(III)TPPS can be used successfully in many situations, contrast enhancement between the organ and tumour in the liver is not achieved. This is probably due to the rapid excretion of the complex through the kidney and urine [168]. Therefore, researchers have developed other synthetic porphyrins which are lipid-soluble, in the hope that these will be excreted through the liver and bile and hence improve imaging of the liver. In a study comparing Mn-mesoporphyrin (Fig. 23) and Mn-TPPS, no enhancement of liver tissue image was seen with Mn-TPPS. With mesoporphyrin, there was significant enhancement of the signal intensity of normal liver parenchyma, enabling contrast-enhanced images of both liver abscesses and metastatic disease within 1 h. It was proposed, from radioactivity studies, that the mesoporphyrin derivative is taken up directly by the hepatocytes where accumulation occurs and is eventually excreted into the bile.

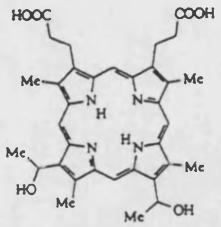
Similar results have been obtained with Mn(III) haematoporphyrin (Fig. 24). However, this complex is retained for a long time in the liver and could give rise to toxic effects [168].

Mn-protoporphyrin IX (Fig. 25) is also targeted towards the liver in rat biodistribution



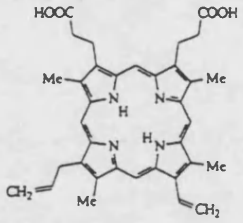
Mesoporphyrin
(77)

Fig. 23.



Haematoporphyrin
(78)

Fig. 24.



Protoporphyrin IX
(79)

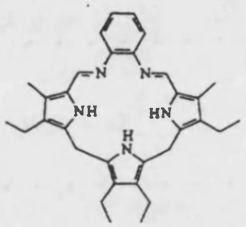
Fig. 25.

studies. However, although it is a very stable complex, it has relatively low relaxivity and is not widely used [166].

Mn-mesoporphyrin has also been used in the imaging of rat glioma models. Increased enhancement of the tumour tissue image was achieved, allowing the tumour to be distinguished from oedema and normal tissue. As with TPPS, the enhancement was prolonged in comparison to Gd-DTPA [169]. One added advantage of using lipid soluble porphyrins is their lack of photosensitisation. With Mn-TPPS, test animals experience both pigmentation of the skin and photosensitivity, with Mn-mesoporphyrin these effects are not seen [168].

The gadolinium ion is too large to form a stable complex with the porphyrin skeleton. However, as the relaxivity of Gd^{3+} is higher there have been attempts to exploit the targeting properties of porphyrins to deliver Gd complexes. Recently, Hindré et al. [170] developed a conjugate of Gd-DTPA with a non-metallated tetraphenylporphyrin derivative. The relaxivity of this conjugate in vitro was greater than that of Gd-DTPA, probably owing to the increased size and hence increased correlation time. Enhanced

images of human adenocarcinoma implanted into mice were achieved after 24 h, demonstrating an increased tumour uptake of the Gd ions due to the tumour specificity of porphyrins. An alternative approach is the use of modified porphyrins, known as texaphyrins, in which there is an expanded central region that contains five rather than four nitrogen atoms, allowing incorporation of Gd ions (Fig. 26) [145].



Texaphyrin
(80)

Fig. 26.

Recently, a new class of metallated cationic porphyrins has been evaluated for targeting to malignant melanoma (Fig. 27) [171]. Although these porphyrins contained radioactive ^{111}In, the results provide information on the potential of targeting paramagnetic ions to one of the most diffuse metastatic cancers. These complexes gave excellent tumour localisation and tumour: blood ratios which were retained for >72 h. Interestingly, it was also possible to see where the porphyrin localised within the tumour. In this case, localisation was seen in the outer growing region, whereas distribution is mainly to the necrotic core with TPPS.

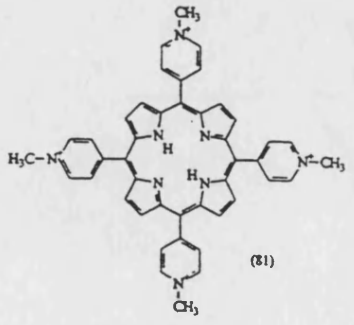


Fig. 27.

6.4. Macromolecules

Proton relaxation enhancement can be achieved by the direct complexation of a paramagnetic ion to a macromolecule or through the attachment of a metal chelator to a macromolecule. The enhancement is due to an increase in the molar relaxivity of the complex. This effect is caused by the slow rotation of the paramagnetic ion which leads to an increased correlation time. The degree of effect depends upon the rigidity of the macromolecule and its tertiary structure. Relaxivities are higher when the paramagnetic ion is complexed with rigid globular protein molecules than with more flexible synthetic poly-(amino acids). The effect of the increased correlation time is highest at higher field strengths where there is a large increase in relaxivity. Both non-covalent and covalent complexes can be prepared. Non-covalent complexes, which result from a combination of electrostatic, van der Waals, hydrogen bonding and hydrophobic interactions, are more stable, through the action of many functional groups on the polymer, and exhibit higher relaxivities. In this situation, the metal is frequently in equilibrium with metal in the bulk solution. On the other hand, although covalent attachments are attractive due to ease of characterisation, proteolytic degradation within lysosomes can lead to release of the paramagnetic ion or chelator resulting in toxicity [172].

Complexation of a paramagnetic ion to a macromolecule has many advantages. Multiple paramagnetic ions can be attached to one large carrier molecule; therefore, the molar dose of imaging agent can be reduced and hence toxicity can be reduced. More importantly, the macromolecularly bound agents can be used to image the blood pool and, hence, situations where vascular integrity is lost, such as in tumours and inflamed tissue [173]. Direct complexation of Gd and Mn to proteins such as concanavalin A and bovine serum albumin results in enhanced molar relaxivities [174]. This is also apparent with the iron-replacement treatment, Imferon, where iron is complexed with dextran. Although it has low magnetic susceptibility, it does display enhanced blood residence times, low toxicity and high

solubility. The synthetic polymer, poly(aspartic acid), has also been used to chelate Gd^{3+} ions directly. Increased relaxivities were demonstrated with chelates bearing <40 ions but, above this level, the relaxivity is reduced. This is probably due to interactions between the chelated ions [172]. Paramagnetic chelates can also be bound non-covalently to albumin. The hepatobiliary contrast agent Fe-EHPG has been successfully bound to albumin leading to a 2–3-fold increase in relaxivity. The binding affinity can be enhanced by increasing the lipophilicity of the chelate [145].

Considerably more interest has been directed towards the development of covalent macromolecular chelate complexes. Albumin is retained within the vascular space after intravascular injection. Conjugates of albumin Gd-DTPA have been used in studies in both rats and rabbits for imaging myocardial ischaemia [175]. In normal rats [176] attachment of the chelate to albumin results in better imaging of the heart, liver and brain at very low doses. Subcutaneous tissue and muscle are not enhanced, unlike with free Gd-DTPA. In this study, sixteen chelates were attached to each albumin molecule and a 3-fold increase in relaxivity of the chelate was achieved on binding. Albumin is a natural polymer with high dispersity and may be immunogenic and exhibits cardiovascular toxicity; thus other macromolecules have also been evaluated.

Dextran is available in a variety of sizes, has low toxicity and can be prepared to contain a biodegradable bond to ensure elimination of bound chelates. Chelation of Gd-DTPA to dextran leads to a large increase in relaxivity which is dependent on the number of chelates bound [177,178]. The chelates are successful in enhancing the imaging of the blood pool in rats but are not as efficient as the albumin chelates. This could be due to the larger molecular weight of albumin or to the higher permeability of the capillaries to dextran. Incorporation of a biodegradable bond enabled both superior imaging and excretion of the chelate in comparison to the free chelate [178]. Interestingly, with a large increase in the molecular weight of dextran, relaxivity is decreased. This is thought to be due to displacement of the bound water molecules from the chelate by neighbouring hydroxyl groups, owing to the polymer wrapping around itself [179].

Synthetic poly(amino acids) have also been used for formation of Gd-DTPA complexes. Poly(Lys)-Gd-DTPA allows enhanced images of the blood pool, pulmonary disease, such as oedema and embolisms, and the heart [176,180]. Mn(III) porphyrins have also been conjugated to poly(amino acids). Cross-linked polymers were produced through coupling of protoporphyrin IX to a range of amino acids, poly(Glu) derivatised with lysine, poly(Lys), poly(Lys-co-Phe) and poly(Lys-co-Ala) [181]. All these conjugates exhibited higher relaxivities than the free porphyrin, possibly owing to decreased porphyrin aggregation as well as the increased rotational correlation time [182]. The highest binding of the porphyrin was achieved with the poly(Glu)-based polymer, which may be due to incorporation of a long spacer unit. However, if the loading factor becomes too high, relaxivity enhancement may be lost owing to interactions between the bound chelates. Attempts by these workers to produce a linear polymer using a monofunctionalised porphyrin resulted in an insoluble polymer [181].

Poly(ethylene glycol) has also been used to change the relaxivity and toxicity of paramagnetic chelates. Desferrioxamine is an effective iron chelator but can lead to hypotensive effects on administration. A non-ionic PEG derivative has been prepared which was non-toxic and allowed enhanced imaging of the kidney and liver in dogs [173].

Recently, it has been suggested that dendrimers or cascade polymers would be more effective relaxation enhancers, owing to their high rigidity and potential for binding a large number of ions. The first branched polymers used were based on polyethyleneimine which can directly chelate paramagnetic ions. However, dendrimers based on Starburst® have also been evaluated [179]. Monodispersed chelates could be produced with varying numbers of chelating ligands, depending on the cycle of polymerisation, allowing between 11 and 170 Gd ions to be bound to the DTPA-dendrimer. The relaxivity of the macromolecule

with 170 Gd ions bound was equivalent to that produced by 1074 free Gd-DTPA molecules and was over 10 times greater than that achieved with linear polymers. Interestingly, no mass effect is seen with these macromolecules; increasing the mass does not lead to a decrease in relaxivity. The wide variety of molecular sizes available with this system leads to a diverse set of applications; small complexes can be used for extravascular imaging of tumours and brain, whereas large complexes can be used for imaging of vasculature.

6.5. Particulates

Imaging of small lesions in liver and spleen is difficult to achieve using MRI. Although images can be enhanced using Gd-DTPA, the enhancement is transitory owing to the fast blood and urinary clearance of the chelate and to the lack of an active uptake mechanism [183]. This can be overcome by the use of higher doses; however, higher and potentially unacceptable toxicity will ensue. An alternative approach is to use either particles or liposomes containing paramagnetic ions or chelates. Liposomes, owing to their size, are retained in vasculature for a prolonged time period and are removed from the circulation either within the lung or by the action of phagocytic cells of the reticulo-endothelial system. In the normal liver, liposomes can be taken up by the Kupffer cells or, if very small, can be taken up by hepatocytes. Hepatic tumours lack phagocytic cells and cannot take up liposomes [184]. Therefore, by preparing liposomes containing contrast enhancement agents, the normal tissue image can be enhanced relative to that of the tumour and a negative image of the tumour can be obtained.

There are three approaches to incorporation of contrast enhancement agents into liposomes. Firstly, a water soluble chelate, such as Gd-DTPA, can be incorporated into the aqueous core of the liposome. Secondly, a lipid-soluble chelate can be incorporated into the pre-formed lipid bilayer. Finally, the chelate can be modified to prepare amphipathic derivatives which can be used, along with other lipids, to form the bilayer [184]. Liposomes have been prepared in which Gd-DTPA is enclosed within the aqueous core [185–188]. These liposomes have been tested for their relaxivity in vitro and stability in serum and saline. Liposomes were prepared in a variety of sizes between 50 and 400 nm in diameter from egg phosphatidylcholine (EPC). The relaxivity of the paramagnetic ion in liposomes was less than for free paramagnetic chelates and was highest in the smallest liposomes which have the largest surface area: volume ratio. It has been postulated that relaxivity of a chelate-containing liposome is directly proportional to the flux of water molecules across the liposomal membrane, which is itself related to the surface area: volume ratio. The loss of image intensity due to decreased relaxivity can be overcome if the agent becomes concentrated in the desired region. With small Gd-DTPA liposomes, there is increased uptake into the liver, vascular space and bone marrow and the body clearance is increased to 3.5 days in mice. This distribution allows imaging of small hepatic metastases with doses as low as 0.025 mmol kg^{-1} of liposome. The acute toxicity of the liposomal preparation is not dissimilar to that of standard chelates. However, on continued administration, the mice treated with the liposomes demonstrated both enlarged livers and spleens. It has been suggested that this effect may not be seen if liposomes containing Gd-DOTA, where the ion is bound more tightly, are used.

The main disadvantage with entrapping the chelate within the aqueous core is the reduced relaxivity. If the paramagnetic ion could be presented to the desired cells on the surface of liposomes, this could be overcome. Thus, a second type of liposome has been prepared in which the chelating ligand is derivatised to be lipid-like, allowing it to be incorporated into the lipid bilayer [188]. The amphipathic chelate Gd-DTPA-SA was prepared and mixed with EPC and cholesterol to form liposomes. Maximal relaxivity was seen with a 33.3% incorporation of the modified ligand; in studies in vitro, the relaxivity of spleen and liver was increased by 110% and 66% respectively. Biodistribution was also evaluated, the liposomes rapidly clearing from the blood and accumulating in liver and

spleen from which they are cleared after eleven days. No toxicity was seen. Following on from this work [186], a second amphipathic ligand has been prepared which would allow more rapid clearance from the liver. Thus, the ligand was bound to the lipid via a stearyl ester (SE) group, rather than through a stearyl amide (SA) group. With a 33.3% incorporation, comparable T_1 signal enhancement was observed as for Gd-DTPA-SA. However, although the liposomes clear as rapidly from the blood and accumulate in the liver, 50% of the liposomes are cleared after 2 days. In a further study [189], liposomes were prepared containing the derivatised ligand DTPA-SA in a variety of concentrations. Gd^{3+}, Mn^{2+} and Fe^{3+} were then incorporated into the chelate and the liposomes were evaluated. The Fe^{3+} liposomes were unstable on formation and could not be evaluated. The Mn^{2+} liposomes were unstable in serum; however, they accumulated rapidly in the liver and were excreted rapidly, allowing good quality images to be obtained. The Gd^{3+} liposomes, in contrast, were stable in plasma, were accumulated rapidly into the liver and were retained, allowing images to be obtained even after 24 h. Thus, liposomes are highly attractive for specific delivery to the liver and spleen. Incorporation of the ligand in either part of the liposome allows enhanced images with lower doses of paramagnetic ions. It has been suggested that this technology could be further exploited. Liposomes can be diverted from the reticulo-endothelial system by using large doses or very small vesicles or by 'blocking' the phagocytic cells by pre-treatment with empty liposomes. These alterations would allow blood pool imaging. Alternatively, targeting moieties could be incorporated into the lipid bilayer to allow cell-specific targeting [184].

It has also been suggested that particles can be used for the carrying of chelates. In a study of cross-linked starch microspheres substituted with DTPA, a variety of paramagnetic ions were coupled and exhibited increased relaxivity over simple chelates. The increase in relaxivity is possibly due to the swelling of the microsphere in water, allowing passage of water protons close to the paramagnetic ions [190]. Microspheres offer potential as contrast enhancement agent carriers and deserve further evaluation.

6.6. Nitroxides, molecular oxygen and nitrogen stable free radicals

Other paramagnetic species, apart from metallic ions, have been evaluated as contrast agents. Molecular triplet oxygen has been used in MRI of the lung through inhalation. In these studies, signal enhancement was seen in the left oxygenated lung in comparison to the right lung. However, this alteration is insufficient to warrant further investigation. A more promising approach, utilising paramagnetic molecules, is the use of stable free radicals or 'spin labels', particularly nitroxyl stable free radicals. Nitroxyls are labile to redox reactions; steric protection confers stability. Then the moiety is relatively unreactive in most conditions and remains stable, even when heated $>100°C$ and under a wide range of pH conditions. These compounds provide strong relaxation enhancement because of electron paramagnetism that is comparable to Cu^{2+} or Fe^{3+} paramagnetic ions. They produce decreases in both T_1 and T_2. However, these compounds offer one major advantage over other paramagnetics, which is that they can be chemically modified through the R group or attached to targeting molecules or biomolecules to allow selectivity of action. Previously, these compounds have been successfully attached to drugs such as propranolol and steroids to study drug metabolism.

Nitrogen stable free radicals have mainly been used as spin probes to study biochemical systems using electron spin resonance spectroscopy. These studies demonstrate that the compounds persist for prolonged periods in biological tissues without electron pairing or reduction taking place by the formation of hydroxylamines [191]. They have been mainly used in MRI to asses renal function and identify renal abnormalities. In an early study [191], the compound TES (*N*-succinyl-4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl) was used to image the kidney and assess renal function in animals with renal ischaemia, congestion or atrophy. The free radical is rapidly

excreted in urine with a clearance rate equal to the glomerular filtration rate. This allows a transient increase in signal intensity in the kidney, permitting increased determination of renal abnormalities. Nitrogen stable free radicals have not been widely used in MRI, as little advantage is obtained over simple paramagnetic chelates. However, one interesting use of nitrogen stable free radicals is in imaging of hypoxic regions of solid tumours. These compounds can be used as probes of oxygen concentration, as in low oxygen reducing environments such as in hypoxia, the compound is reduced and the signal enhancement is lost. [191]

6.7. Superparamagnetic particles

Small ferrite particles have superparamagnetic properties; that is, they are easily magnetised to a high magnetic moment but the magnetism is lost once the external magnetic field is removed. An example of a ferrite particle is magnetite, a natural compound Fe_3O_4, which is often found in combination with maghaematite and haematite Fe_2O_3. Superparamagnetic particles exert effects on both T_1 and T_2, but the effect on T_2 outweighs all other effects leading to a loss of signal in the affected organs [192]. Magnetite particles are administered as polymer coated particles to allow the formation of aqueous solutions, and alter particle size. Although both starch and albumin have been used, the majority of studies have been performed on AMI-25, a commercial product, in which the iron oxide is coated in dextran [145]. Initially, studies were carried out using relatively large particles designed to be captured by the cells of the reticuloendothelial system allowing imaging of the liver and spleen [193]. AMI-25 has been used in clinical trials for the diagnosis of liver cancer [194,195], cerebral vasculature [196] and cardiovascular imaging [197]. Studies [195] in 15 patients with known liver cancers demonstrated hyperintense imaging of these lesions. Normal liver cells, i.e., Kupffer cells take up the particles by phagocytosis and there is a loss of signal in these cells and the surrounding hepatocytes giving a negative image of the liver. Abnormal cells loose the ability to phagocytose, so do not take up the contrast agent and thus appear hyperintense on the image. Although 90% of the particles were cleared by the liver in 1 h, some remains circulating allowing imaging of other systems, particularly the heart [197] and brain [198]. Clearance from the liver is demonstrated in humans within 24 h of treatment [195]. Breakdown of the particle does not lead to abnormalities or toxic effects.

Studies with microspheres, in which magnetite is imbedded in an albumin matrix, have also demonstrated enhanced imaging of hepatic tumour models in rabbits. These are slightly larger particles (1–5 μm) but they are still efficiently taken up by the reticulo-endothelial system and are rapidly cleared from the blood stream. There is long-term retention of these microspheres. However, no toxicity or immunogenicity has been demonstrated in that, after two months, there is no evidence of hepatocellular damage or fibrosis [199]. Ultrasmall dextran-magnetite particles have also been developed by particle size sorting of AMI-25. In normal rats [200] over 24 h, accumulation was higher in the lymph nodes, bone marrow, liver and spleen and the blood half-life was increased from 6 min for AMI-25 to 81 min. These particles are small enough to transmigrate through capillaries and are taken up by the lymphatics. Without contrast enhancement, there is a considerable overlap in the relaxivities of both normal and metastatic lymph tissue. However, on administration of ultrasmall particles, metastatic lymph tissue is visualised as hyperintense regions, in comparison to normal tissue, as it cannot phagocytose [201].

With these developments have come further approaches to targeting, away from the reticuloendothelial system. One approach is the ferrosome; this is a small vesicle that contains superparamagnetic particles in the central aqueous compartment. These have been evaluated in the imaging of lymph nodes, pulmonary emboli, adenocarcinomas and hepatomas. In tumour tissue, they are taken up by the macrophages located at the surface of the tumour giving a rim of low signal intensity. Alternatively, the superparamagnetic particles can be coated in polymers

with intrinsic targeting properties. Particles coated with arabinogalactose can be targeted to hepatocytes via the asialoglycoprotein. This achieves imaging of the liver with reduced doses in comparison to targeting to Kupffer cells.

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PUBLICATION 78

Poly(ADP-ribose)polymerase Inhibitors - Radiosensitizers and Chemosensitizers

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123. POLY(ADP-RIBOSE)POLYMERASE INHIBITORS RADIO- AND CHEMO-SENSITISERS

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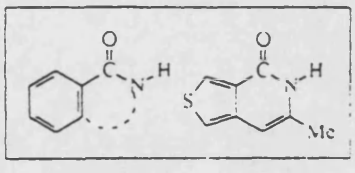

The nuclear enzyme poly(ADP-ribose)polymerase (PARP) catalyses transfer of ADP-ribose from NAD^+ to nucleophilic acceptor sites on DNA-associated proteins in response to local strand breaks. A polyanionic polymer is built up by attachment of ADP-ribose units to the growing chain. The poly(ADP-ribose) is implicated in several processes, including DNA repair, gene expression and apoptosis. Inhibition of PARP in tumour cells leads to persistence of DNA damage and to potentiation of the cytotoxic effects of ionising radiation and chemotherapeutic drugs. Known competitive reversible inhibitors of PARP are analogues of the nicotinamide moiety of NAD and include 3-aminobenzamide, 5-substituted isoquinolinones and quinazolinones, all containing the consensus conformationally constrained arenecarboxamide structure. Recent work in our group has been focussed on development of PARP inhibitors which are selective for hypoxic tumour cells and/or are mechanism-based irreversible inhibitors. Further, we have shown that some heteroarylcarboxamides and conformationally constrained heterobicycles are potent inhibitors.

PUBLICATION 79

Synthetic Approaches to Irreversible Inhibition of Poly(ADP-ribose)polymerase

C. Y. Watson, M. D. Threadgill and W. J. D. Whish

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231A SYNTHETIC APPROACHES TO IRREVERSIBLE INHIBITION OF POLY(RIBOSE) POLYMERASE

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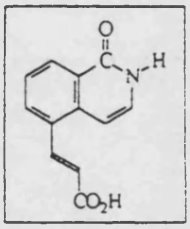
Radiosensitisation and chemosensitisation of tumour cells can be effected by inhibition of DNA repair. Excision repair of DNA damaged by radiation or alkylating agents is triggered by poly-(ADP-ribosyl)ation of histones near the damaged site. The enzyme responsible, poly(ADP-ribose) polymerase (PARP), uses NAD^+ as the source of ADP-ribosyl units. We are currently synthesising and testing potential mechanism-based suicide inhibitors of PARP in the form of 3-substituted benzamides and 5-substituted isoquinolinones. As expected, the 5-substituted isoquinolinones have shown much greater inhibition of PARP than the benzamides tested thus far. For example, 5-bromoisoquinolinone caused >95% inhibition of PARP activity in isolated nuclei at 11 μM. This is due to the fixed conformation of the amide group in the isoquinolinones. Recent success in the synthesis of 5-iodoisoquinolinone has led to the development of isoquinolinones with more complex side chains, *via* the Heck coupling reaction.



PUBLICATION 80

Synthesis of Potential Bioreductively Activated Inhibitors of DNA Repair

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231B SYNTHESIS OF POTENTIAL BIOREDUCTIVELY-ACTIVATED INHIBITORS OF DNA REPAIR

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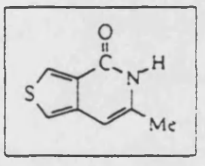

One approach to increase the selectivity of radiation and drugs for cancer cells is to exploit the lower oxygen concentration in the core of poorly vascularised solid tumours, by designing prodrugs which are metabolically activated to inhibitors of poly(ADP-ribose)polymerase (PARP) in this environment. PARP uses NAD^+ as the source of ADP-ribosyl units for poly(ADP-ribosyl)ation of histones near a site of damage in DNA. Thus, inhibition of this enzyme will impede DNA repair. 3-Aminobenzamide and 5-substituted isoquinolinones, analogues of NAD$^+$, inhibit PARP. The synthesis and evaluation of a series of nitro- and aminothiophene analogues of these inhibitors has been undertaken. A large differential is required between the inhibitory activity of the nitro pro-drug and the active drug, the amino metabolite. Studies using isolated cell nuclei showed >80% inhibition for the thieno-pyridinone (above) at 10 μM and >60% inhibition at 12 μM for thiophene-2-carboxamide. The isomeric thiophene-3-carboxamide had previously been reported to be inactive. Good selectivity has been shown between the activity of pairs of nitro- and amino-thiophene carboxamides.

PUBLICATION 81

S-2-Amino-5-(2-nitroimidazol-1-yl)pentanoic acid: A Model for Potential Bioreductively Activated Prodrugs for Inhibitors of Nitric Oxide Synthase (NOS) Activity

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Anti-Cancer Drug Design (1997), 12, 61–65

S-2-Amino-5-(2-nitroimidazol-1-yl)pentanoic acid: a model for potential bioreductively activated prodrugs for inhibitors of nitric oxide synthase (NOS) activity

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Summary: Treatment of 1,1-dimethylethyl *S*-(2-1,1-dimethylethoxycarbonylamino)-5-bromopentanoate with 1-potassio-2-nitroimidazole, followed by deprotection, afforded *S*-2-amino-5-(2-nitroimidazol-1-yl)pentanoic acid, which was reduced to *S*-2-amino-5-(2-aminoimidazol-1-yl)pentanoic acid. This aminoimadazole inhibited rat brain nitric oxide synthase (NOS) activity 3.2 times more potently than did the nitro analogue. Thus *S*-2-amino-5-(2-nitroimidazol-1-yl)pentanoic acid is a potent prodrug which may be bioreductively activated to a NOS inhibitor in hypoxic solid tumours.

Key words: bioreductive/hypoxia/nitroimidazole/prodrug

Introduction

One site of the messenger activity of nitric oxide (NO) is the vascular endothelium, where it is a vasodilator and is partly responsible for maintaining cardiovascular homeostasis (Moncada *et al.*, 1991). NO is synthesized from L-arginine by nitric oxide synthase (NOS), yielding L-citrulline as a co-product. The process involves two separate mono-oxygenation steps, with N^G-hydroxyarginine as an intermediate, through a reaction requiring O_2 and NADPH. The isoforms of NOS are in two groups: a constitutive, Ca^{2+}/calmodulin-dependent type (cNOS) and an inducible, Ca^{2+}/calmodulin-independent form (iNOS). Known inhibitors of the enzyme include N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine (NOARG) (Moore *et al.*, 1990; Rees *et al.*, 1990). NOS inhibitors that have particular tissue or isozyme specificities open up a variety of therapeutic possibilities (Marletta, 1994). Recently, NOS inhibitors have been used selectively to modulate tumour blood flow, oxygenation and redox status (Andrade *et al.*, 1992; Wood *et al.*, 1993, 1994).

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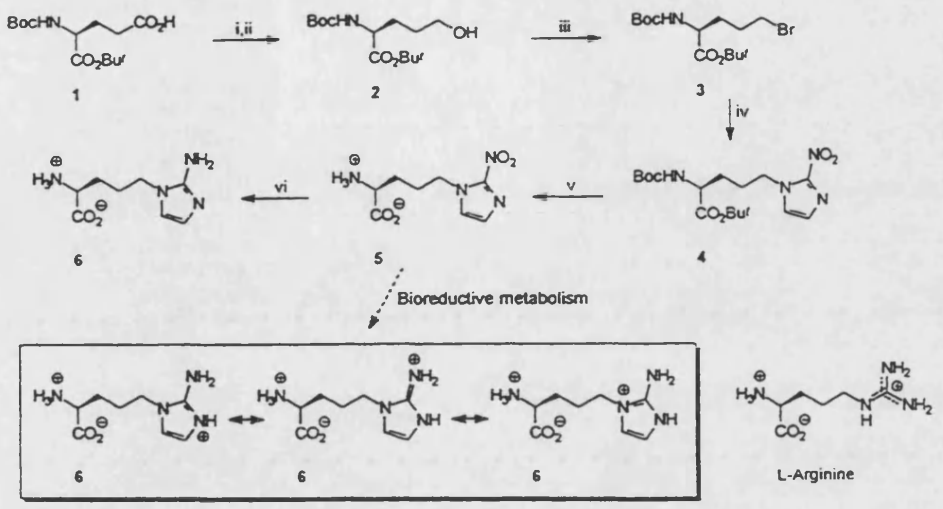

Scheme 1 Synthesis of prodrug 5 and aminoimidazole 6, and the relationship of the protonated form of 6 to the structure of L-arginine. Reagents and conditions: (i) $ClCO_2Et/Et_3N$; (ii) $NaBH_4$; (iii) $CBr_4/Ph_3P/THF$; (iv) 1-potassio-2-nitroimidazole/DMF, reflux, 2 h, 76%; (v) HCl/water/EtOAc then chromatography in the presence of NH_3, 81%; (vi) H_2/EtOH/Pd/C, 83%.

However, the inhibitors used (L-NMMA and NOARG) also have systemic effects related to their effects on NO production. Almost unique to most human and experimental solid tumours are regions of hypoxic tissue (Vaupel *et al.*, 1991; Wilson, 1992) which have been used as targets for selective bioreductive activation of prodrugs (Workman & Stratford, 1993; Adams & Stratford, 1994). Thus, prodrugs that yield NOS inhibitors following hypoxia-mediated reductive activation could provide a novel strategy for selective vascular shut-down in tumours, while avoiding systemic effects. 2-Aminoimidazole analogues of L-arginine are an attractive class of potential inhibitors, since protonation gives a guanidinium-like cationic system which is formally very similar to that of arginine (Scheme 1). 1-Substituted 2-nitroimidazoles can undergo bioreductive metabolism to give 2-aminoimidazoles (Walton & Workman, 1987; McClelland, 1990), presenting a potential strategy for developing prodrugs that, following hypoxia-mediated bioreduction in tumours, will give species that could inhibit NOS and thereby produce local vascular shutdown. Here we examine the first step in this strategy in describing the synthesis of novel 2-nitro- and 2-aminoimidazole analogues of L-arginine and their evaluation as inhibitors of cNOS and iNOS obtained from the brain and lung, respectively, of rats treated with lipopolysaccharide (LPS).

Chemistry

Following the method of Olsen *et al.* (1984), the γ-carboxylic acid of BocGluOBu^t 1 was converted to the mixed anhydride with ethyl chloroformate. Selective reduction to the corresponding alcohol 2 was effected with sodium borohydride. Treatment with

triphenylphosphine and tetrabromomethane then served to replace the alcohol to form the corresponding bromo compound 3 in good yield (Olsen *et al.*, 1984). This material 3 was used to alkylate 1-potassio-2-nitroimidazole in good yield under the usual conditions (Scobie & Threadgill, 1994). Acidolytic deprotection gave the prodrug 5 which was converted to the amino analogue 6 by reduction with hydrogen in the presence of palladium.

Materials and methods

Chemical synthesis

1,1-Dimethylethyl S-N-(1,1-dimethylethoxycarbonylamino)-5-(2-nitroimidazol-1-yl)pentanoate (4). The protected bromo amino acid 3 (Olsen *et al.*, 1984) (560 mg, 1.6 mmol) was boiled under reflux with 1-potassio-2-nitroimidazole (340 mg, 3.0 mmol) in dimethylformamide (40 ml) for 2 h. Evaporation and chromatography (silica gel, ethyl acetate/hexane 1:1) gave 4 (550 mg, 76%) as a white solid: mp 83–85°C; found: C, 53.0; H, 7.2; N, 14.4. $C_{17}H_{28}N_4O_6$ requires C, 53.1; H, 7.3; N, 14.6. ^{1}H NMR ($CDCl_3$) δ 1.47 (18 H, s, 2 × Bu^t), 1.97–2.10 (4 H, m, 3-H_2 + 4-H_2), 4.60 (1 H, m, 2-H), 4.96 (2 H, m, 5-H_2), 5.38 (1 H, m, NH), 7.14 (2 H, s, imidazole 4,5-H_2); mass spectrum (FAB positive ion) 407 (M + Na), 386.2136 (M + H) ($^{12}C_{16}{}^{13}CH_{29}N_4O_6$ requires 386.2121), 385.2088 ($^{12}C_{17}H_{29}N_4O_6$ requires 385.2087); mass spectrum (FAB negative ion) 384 (M).

S-2-Amino-5-(2-nitroimidazol-1-yl)pentanoic acid (5). Compound 4 (520 mg, 1.4 mmol) was stirred with hydrochloric acid (4 M, 30 ml) and ethyl acetate (30 ml) for 4 h. Evaporation and chromatography (silica gel, methanol/35% aqueous ammonia 49:1) gave 5 (300 mg, 81%) as a hygroscopic white solid: mp 178–181°C; ^{1}H NMR (D_2O) δ 1.9–2.2 (4 H, m, 3-H_2 + 4-H_2), 4.14 (1 H, t, J = 5.3 Hz, 2-H), 4.54 (2 H, t, J = 6.0 Hz, 5-H_2), 7.21 (1 H, s) and 7.51 (1 H, s) (imidazole 4,5-H_2); ^{13}C NMR δ 28.07, 29.51, 52.18, 55.25, 130.67, 130.91, 147.16, 174.47; mass spectrum (FAB positive ion) *m/z* 229.0930 (M + H) ($C_8H_{13}N_4O_4$ requires 229.0937).

S-2-Amino-5-(2-aminoimidazol-1-yl)pentanoic acid (6). Compound 5 (700 mg, 2.6 mmol) in ethanol (40 ml) was treated with hydrogen in the presence of 10% palladium on carbon (50 mg). After 2 h, the solution was filtered through Celite® and the solvent was evaporated. Recrystallization (methanol/diethyl ether) gave 6 (520 mg, 83%) as a hygroscopic white solid: mp 174–176°C; ^{1}H NMR (D_2O) δ 1.9–2.1 (4 H, m, 3-H_2 + 4-H_2), 3.94 (2 H, t, J = 6 Hz, 5-H_2), 4.10 (1 H, t, J = 6 Hz, 2-H), 6.84 (1 H, s) (imidazole 4,5-H_2); ^{13}C NMR δ 26.70, 29.52, 47.33, 55.47, 115.65, 119.18, 148.64, 174.72; mass spectrum (FAB positive ion) *m/z* 199.1202 (M + H) ($C_8H_{15}N_4O_2$ requires 199.1195).

Biological evaluation and conclusions

Compounds 5 and 6 were evaluated as inhibitors of NOS activity, based on the conversion of L-[U-^{14}C]arginine to L-[U-^{14}C]citrulline and NO, using the method of Salter *et al.* (1991). Male Wistar rats (200–300 g), fed *ad libitum*, were injected i.p. with 4 mg/kg trichloroacetic acid-extracted LPS (Knowles *et al.*, 1990). After 6 h, the rats were killed and the brain and lungs were removed. Cytosolic fractions of rat brain and lung were used as the source of cNOS and iNOS respectively (Knowles *et al.*, 1990). Figure 1 shows the concentration–inhibition curves for the effects of 5 and 6 on cNOS from rat brain. Both

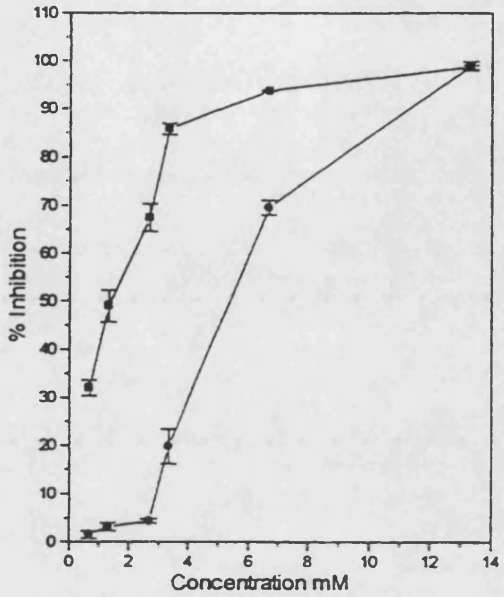

Figure 1 Concentration–response curves for inhibition of rat brain cNOS by 5 (■) and 6 (●). Values are expressed as mean ± SEM (n = 9), obtained from three separate determinations, each performed in triplicate.

compounds are inhibitory, with IC_{50} = 1.7 mM for the amine **6** and IC_{50} = 5.3 mM for the nitro prodrug **5**, giving a ratio of potencies of 3.2. These compounds also inhibit iNOS from LPS-induced rat lung, with a similar dose-dependence (data not shown). However, the NO_2/NH_2 differential (2.2) is smaller than that obtained for the Ca^{2+}-dependent form of NOS; the compounds are also slightly less potent against this isoform (**5**: IC_{50} = 6.6 mM; **6**: IC_{50} = 3.0 mM). These results can therefore be regarded as supporting the principle of hypoxia-selective prodrugs of NOS inhibitors with eventual applications in selective modulation of tumour blood flow after bioreduction. The principle of a differential NOS inhibition was our goal and has been demonstrated. The compounds are not potent inhibitors (cf. *S*-N^G-nitroarginine methyl ester: IC_{50} = 7 μM in this system); we are currently investigating modifications to the substituent pattern on the imidazole and their effects on the prototropic equilibria thereof in order to increase potency. Work is also underway to determine whether **5** is reduced in hypoxic tissues, to measure cellular uptake and to establish the ability of **5** or **6** to inhibit NOS activity *in vivo*.

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PUBLICATION 82

5-Nitrofuran-2-ylmethyl Group as a Potential Bioreductively Activated Prodrug System

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5-Nitrofuran-2-ylmethyl group as a potential bioreductively activated pro-drug system

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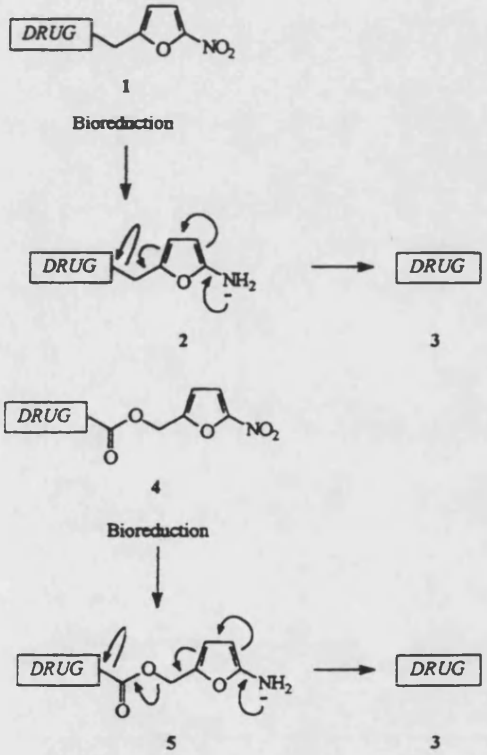
5-Substituted isoquinolin-1-ones have been synthesised by one-pot Curtius rearrangement of the corresponding substituted 3-phenylpropenoyl azides and cyclisation. Arylmethylation of the anions of the isoquinolinones with benzyl halides [4-methoxybenzyl chloride, 2-(chloromethyl)furan and 5-nitro-2-(tosyloxymethyl)furan] takes place exclusively at nitrogen. Nitration of 2-(furan-2-ylmethyl)isoquinolin-1-one in strongly acidic medium gives 2-(5-nitrofuran-2-ylmethyl)isoquinolin-1-one, whereas weaker acidic conditions lead to dinitration. Curtius rearrangement of 3-carboranylbutanoyl azide and trapping with 5-nitrofuran-2-ylmethanol gives 5-nitrofuran-2-ylmethyl *N*-(3-carboranylpropyl)carbamate. Biomimetic reduction of these nitrofuranylmethyl derivatives of anticancer drugs triggers release of the parent drugs. Thus, these nitrofurans have potential applications as pro-drugs for selective release of therapeutic drugs in hypoxic solid tumours.

Regions of chronic and acute hypoxia are present in most solid tumours owing to the primitive state of the tumour vasculature.[1] Viable cells in such tissue are relatively resistant to radiotherapy and to many chemotherapeutic strategies.[1] Much effort has been expended[2] on development of radiosensitisers with electron-affinity and bioreductively activated cytotoxins for selective therapy of this tissue, and of a variety of pro-drugs to deliver cytotoxins selectively to tumours. 1-Substituted 2-nitroimidazoles are known[3,4] to be selectively retained in hypoxic tissue by reductive metabolism. However, relatively little attention has hitherto been focussed on exploiting the physiological difference in concentration of molecular oxygen between normal and hypoxic tumour tissue by design of biologically inactive pro-drug systems which, upon selective bioreduction in hypoxic tissue, would release known therapeutic drugs only in that tissue. This would improve greatly the selectivity of biodistribution of such agents. Sykes *et al.* have reported[5] early studies on a bioreductively triggered release system based on 2-nitroarylamides, whereas 4-nitrobenzyloxycarbonyl pro-drugs have been put forward[6] for use in the Antibody-Directed Enzyme Prodrug Therapy (ADEPT) strategy, using a bacterial nitroreductase attached to a tumour-selective antibody. For the potential pro-drugs described here, 2-nitrofuran was selected as the redox-sensitive moiety. The redox potential of this heterocycle is relatively high [E^1_7 = −325 mV for 2-methyl-5-nitro-*N*-(prop-2-enyl)furan-3-carboxamide],[7] which would favour selective reductive metabolism in hypoxic tumour tissue effected by endogenous enzymes such as cytochrome P450 reductase.[8] The general design of the pro-drugs and the mechanisms of bioreductively triggered release are shown in Scheme 1. For the simpler pro-drugs **1**, the 5-nitrofuran-2-ylmethyl unit is attached to a heteroatom in the drug. Reduction in hypoxic tumour tissue will give the corresponding aminofuran **2** (or the analogous hydroxylamine). The presence of an available electron pair will then promote fragmentation as shown to release the drug **3** in the tumour tissue. This fragmentation is clearly not available to the nitrofuran **1**. Where appropriate, the nitrofuranylmethyl unit could be linked to the drug by an additional readily fissile group, as in the carbamate pro-drugs **4**. Bioreductively triggered fragmentation will again afford **3** according to the mechanism shown. Here



Scheme 1 Proposed mechanisms of bioreductively triggered release of drugs from general nitrofuranylmethyl pro-drugs **1** and **4**

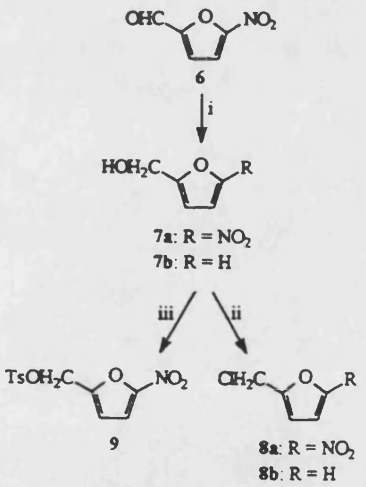
we report syntheses of examples of pro-drugs of each type (**1** and **4**) and biomimetic studies on the release of parent drugs **3**.

Isoquinolin-1-one **18d** and several 5-substituted analogues are potent inhibitors[9] of poly(ADP-ribose)polymerase (PARP), an enzyme with a central role in initiating excision repair of DNA following damage by radiation or electrophilic drugs. Thus inhibitors of PARP are potentiators of these

therapeutic strategies. Prodrugs which selectively release isoquinolinones in hypoxic tumour tissue would therefore act as tumour-selective radiosensitisers. In the design of these prodrugs, it is necessary to conceal the arylcarboxamide motif, with the N–H held *syn* to the carbonyl oxygen, which is essential for enzyme inhibitory activity.[9,10] Within the context of the nitrofuranylmethyl system, this could be achieved through either 1-(5-nitrofuran-2-ylmethoxy)isoquinolines or 2-(5-nitrofuran-2-ylmethyl)isoquinolin-1-ones, pro-drugs of general type **1**.

To form examples of pro-drugs of type **4**, with the carbamate link, 2-phenylethylamine was selected as a model and a carboranylalkylamine was selected as a drug for delivery. Boron neutron capture therapy (BNCT) is under active investigation for the treatment of various cancers, notably gliomas and melanomas.[11] When the ^{10}B isotope is irradiated with slow ('thermal') neutrons, an [n,α] reaction ensues, giving ^{7}Li and ^{4}He nuclei with kinetic energy (2.31 MeV). With this energy, the α-particle has a range of *ca.* one cell diameter in biological tissue and damage is limited to the cell containing the boron. Early clinical failures of BNCT were attributed[12,13] to inadequate concentrations of ^{10}B in the tumour tissue or to lack of selectivity of disposition of ^{10}B, leading to damage of normal tissue. Carboranes have been linked to nucleosides,[14] to porphyrins[15] and to nitroimidazoles[4,16,17] in attempts to target boron selectively to tumours. Where a 5-nitrofuran-2-ylmethyl *N*-(carboranylalkyl)carbamate forms pro-drug **4**, selective bioreduction in a hypoxic tumour cell would release a carboranylalkylamine, which, at the relatively acidic pH of a tumour cell, would be protonated and hence unable to diffuse out through the cell membrane. Boron would therefore accumulate in the tumour tissue.

To provide nucleophilic and electrophilic reagents to introduce the nitrofuranylmethyl group into the pro-drugs, the alcohol **7a** and the nitrofuranylmethyl electrophiles **8a** and **9** were prepared (Scheme 2). Attempts to reduce 5-nitrofuran-2-



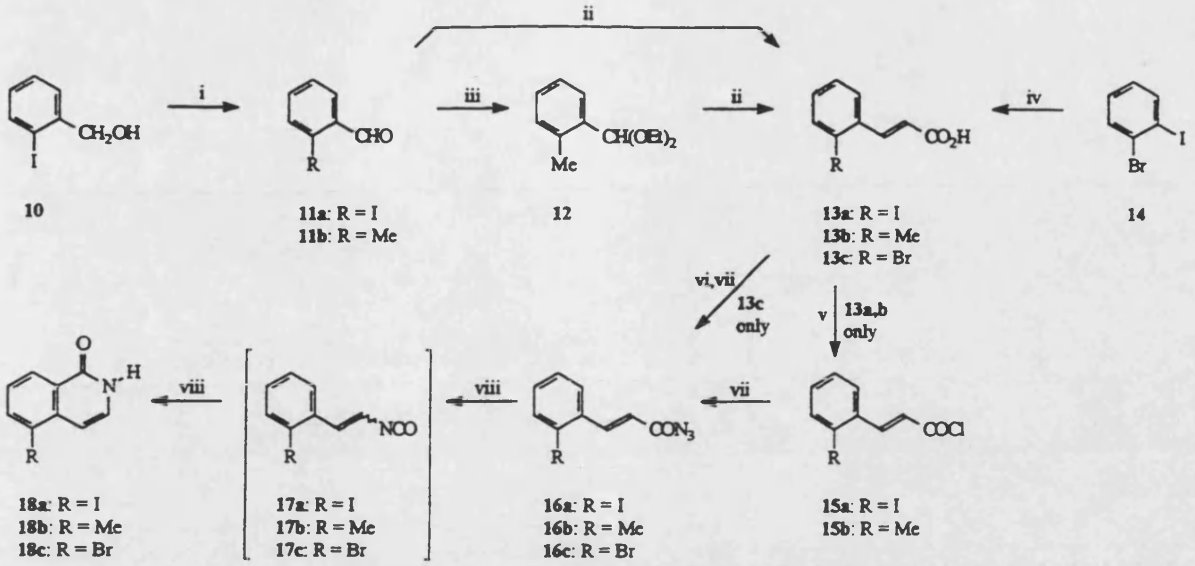
Scheme 2 Synthesis of electrophilic (nitro)furan-2-ylmethyl reagents **8a,b** and **9**. *Reagents:* i, $Al(OPr^i)_3$, Pr^iOH; ii, $SOCl_2$, pyridine, $CHCl_3$; iii, TsCl, KOH, THF.

carbaldehyde **6** selectively at the aldehyde using sodium borohydride were unsuccessful, leading only to products of degradation, in contrast to a previous report.[18] However, Meerwein–Ponndorf–Verley reduction gave the required nitrofuranylmethanol **7a** almost quantitatively. Activation of the methylene as an electrophile was attempted through conversion into the corresponding chloromethylnitrofuran **8a** and the tosylate **9**, respectively. Replacement of OH with Cl to give **8a** was achieved by treatment of **7a** with thionyl chloride and pyridine in a modification of the method of Wang *et al.*[19] Reaction of **7a** with tosyl chloride in the presence of powdered potassium hydroxide afforded the tosylate **9** in modest isolated yield.

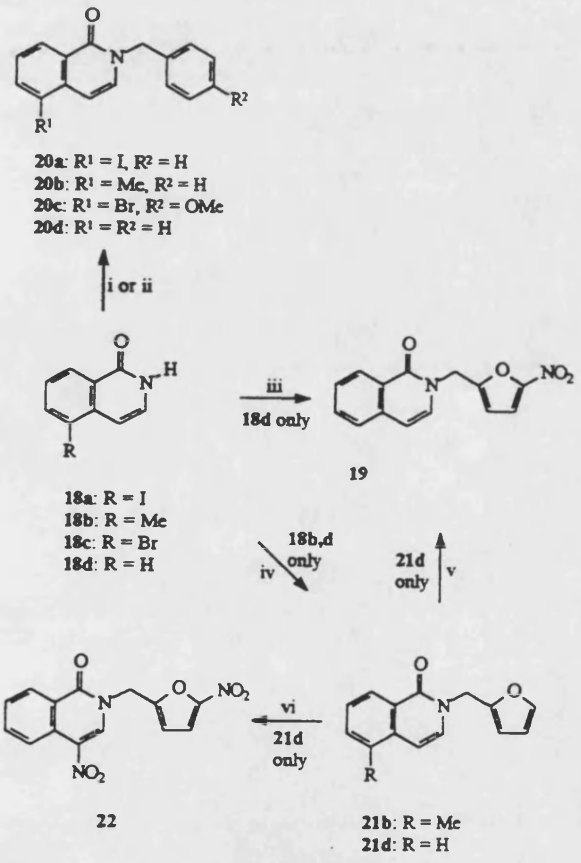
1-Alkoxyisoquinolines have been prepared by substitution in the corresponding 1-chloroisoquinolines using sodium alkoxides under vigorous conditions, although the range of 1-alkoxy substituents reported to have been introduced in this way has been restricted to simple examples,[20] such as methoxy and ethoxy. 1-Benzyloxyisoquinoline has not been reported. Treatment of 1-chloroisoquinoline[21] with 5-nitrofuran-2-ylmethanol **7a** and furan-2-ylmethanol **7b** alkoxides gave only products of furan degradation under a variety of reaction conditions, making the corresponding pro-drugs unavailable by this route.

The usual route for preparation of 2-substituted isoquinolin-1-ones has been oxidation of 2-substituted isoquinolinium salts in the presence of hydroxide ion.[22] However, this is inappropriate for preparation of the proposed pro-drugs, owing to the harsh reaction conditions required. Routes employing *N*-alkylation of isoquinolin-1-ones were therefore investigated. A convenient synthesis of isoquinolin-1-ones by Curtius rearrangement of cinnamyl azides and thermal cyclisation, in a one-pot process, has been described by Eloy and Deryckere,[23] although this is limited to isoquinolinones without electron-withdrawing substituents. 2-Iodobenzyl alcohol **10** was oxidised to the corresponding aldehyde **11a** with pyridinium dichromate and a direct condensation with malonic acid afforded the iodocinnamic acid **13a** (Scheme 3). 2-Methylbenzaldehyde **11b** had to be converted into its diethyl acetal **12** for efficient condensation under the Knoevenagel–Doebner conditions[24] to give the methylcinnamic acid **13b**. The corresponding bromocinnamic acid **13c** was prepared from 2-bromoiodobenzene in a convenient modification of the iodine–selective Heck reaction conditions used by Plevyak *et al.*[25] Use of boiling propionitrile as the reaction solvent obviated the need for the sealed tube required to conduct the reaction at 100 °C in acetonitrile. Various modifications of the original conditions of Eloy and Deryckere[23] were investigated for the Curtius rearrangement and cyclisation sequence. The iodocinnamic acid **13a** was converted into its acid chloride **15a** and treatment with sodium azide furnished the acid azide **16a**. For ease of isolation of the product 5-iodoisoquinolin-1-one **18a** from the reaction mixture by precipitation with water, the Curtius rearrangement and cyclisation were effected in boiling dry tetraglyme. The acid chloride **15b** and acid azide **16b** of the methylcinnamic acid were prepared similarly, but the original boiling diphenyl ether was found to be the optimum solvent for the high-yielding synthesis and isolation of 5-methylisoquinolin-1-one **18b**. The bromoisoquinolinone **18c** was prepared similarly but in poor yield, using the acid azide **16c** formed from a mixed anhydride.

The relatively few reports[26] of alkylation of isoquinolinones indicate that alkylation of isoquinolin-1-ones with simple halogeno alkanes occurs predominantly at the nitrogen of the conjugate anion under a variety of conditions. Two sets of conditions were used in a series of model experiments designed to establish the site of reaction of isoquinolinone anions with halogenomethyl arenes and to optimise the reaction conditions. The anion formed from 5-iodoisoquinolinone **18a** and lithium hexamethyldisilazide was benzylated in high yield by benzyl chloride, giving **20a**. The 5-bromo analogue **18c** was also converted into the *N*-(4-methoxybenzyl) derivative **20c** under these conditions. Benzylation of the sodium anion of isoquinolinone **18d** with benzyl bromide in DMF was also highly efficient in forming **20d**, although the yield in the corresponding benzylation of 5-methylisoquinolinone **18b** to give **20b** was poor. However, treatment of the anion derived from reaction of isoquinolin-1-one **18d** with sodium hydride and with lithium hexamethyldisilazide with the chloromethylnitrofuran **8a** failed to give the required *N*-(nitrofuranylmethyl)isoquinolinone **19**. Compound **19** was formed in poor isolated yield (24%) when the sodium salt of **18d** was treated with the more reactive tosylate **9** in



Scheme 3 Synthesis of isoquinolin-1-ones **18a–c**. *Reagents:* i, pyridinium dichromate, CH_2Cl_2; ii, $CH_2(CO_2H)_2$, piperidine, pyridine; iii, $HC(OEt)_3$, $SOCl_2$, EtOH; iv, $H_2C{=}CHCO_2H$, $Pd(OAc)_2$, Et_3N, EtCN; v, $SOCl_2$, DMF; vi, EtO_2CCl, Et_3N, Me_2CO; vii, NaN_3, water, 1,4-dioxane or acetone; viii, heat, Ph_2O or $(MeOCH_2CH_2OCH_2CH_2)_2O$.



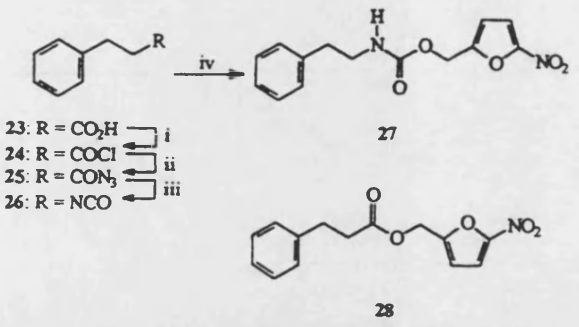
Scheme 4 Synthesis of 1-(arylmethoxy)isoquinolines **20a–d**, 2-(furan-2-ylmethyl)isoquinolin-1-ones **21b,d** and 2-(5-nitrofuran-2-ylmethyl)-isoquinolin-1-one **19**. *Reagents:* i, $LiN(SiMe_3)_2$, BnCl, THF or NaH, BnBr, DMF; ii, $LiN(SiMe_3)_2$, 4-$MeOC_6H_4CH_2Cl$, THF; iii, NaH, **9**, DMF; iv, $LiN(SiMe_3)_2$, **8b**, THF; v, CF_3CO_2H, HNO_3 or $Cu(NO_3)_2$; vi, *e.g.* HNO_3, HOAc.

DMF. Assignment of the structures of **20a,b,d** and **21b,d** as being the *N*-(arylmethyl)isoquinolin-1-ones rather than the 1-(arylmethoxy)isoquinolines was made by ^{13}C NMR spectroscopy and, in the case of **20a**, by IR spectroscopy. The CH_2 groups resonated at δ 51.90, 51.68, 51.59, 44.33 and 44.16, respectively, values which correspond closely to those typical for $ArCH_2N$ but not $ArCH_2O$. The IR spectrum of **20a** contained a band at 1650 cm^{-1}, indicating a carbonyl group. The structure of **20c** was assigned by analogy. Since the target nitrofuran **19** prepared by alkylation of **18d** with **9** was identical with material formed by selective nitration of **21d**, the nitrofuranylmethylation must also have taken place at *N*, rather than at *O*.

As the combined yield over two steps **7a**→**9**→**19** was very low (6%), an alternative longer route was developed. Reaction of the lithium salt of isoquinolin-1-one **18d** with freshly prepared unstable chloromethylfuran **8b**[27] in THF gave a moderate yield of the *N*-furanylmethylisoquinoline **21d**. The 5-methyl analogue **21b** was formed similarly. Selective nitration at the furan 5-position was then required to form **19**. The usual conditions for nitration of furans and related heterocycles are relatively mild, *e.g.* acetyl nitrate or nitric acid in acetic acid. However, all applications of these and other relatively weakly acidic nitrating conditions gave only the dinitrated product **22** where the isoquinolinone 4-position has also reacted. In an extensive study of substitution of isoquinolin-1-ones, Horning *et al.*[28] reported that the principal site of reaction of various electrophiles was the 4-position. However, Kawazoe and Yoshioka[29] noted that, on treatment of **18d** with potassium nitrate in concentrated sulfuric acid, nitration took place at the 5- and 7-positions, presumably owing to deactivation of the heterocyclic ring by protonation. Adopting this approach to selective deactivation of the nitrogen heterocycle, the reaction of a solution of **21d** in trifluoroacetic acid with nitric acid or, preferably, copper(II) nitrate at low temperature effected selective mononitration on the furan, giving the target *N*-(nitrofuranylmethyl)isoquinolinone **19**. Traces of the dinitro compound **22** were also isolated but the major by-product was the parent isoquinolinone **18d** resulting from dealkylation under the acidic conditions. The 5-methyl analogue **21b** gave the parent 5-methylisoquinolinone **18b** as the only isolable product. Thus the sequence **7b**→**8b**→**21d**→**19** proceeded in higher overall yield (16%) than the more direct sequence above.

Since it was planned to form the carbamate link in the target carborane-nitrofuran **38** by addition of 5-nitrofuran-2-ylmethanol **7a** to an appropriate isocyanate, model reactions for the Curtius rearrangement and addition were investigated. The model sequence, in which phenyl replaces carboranyl, also

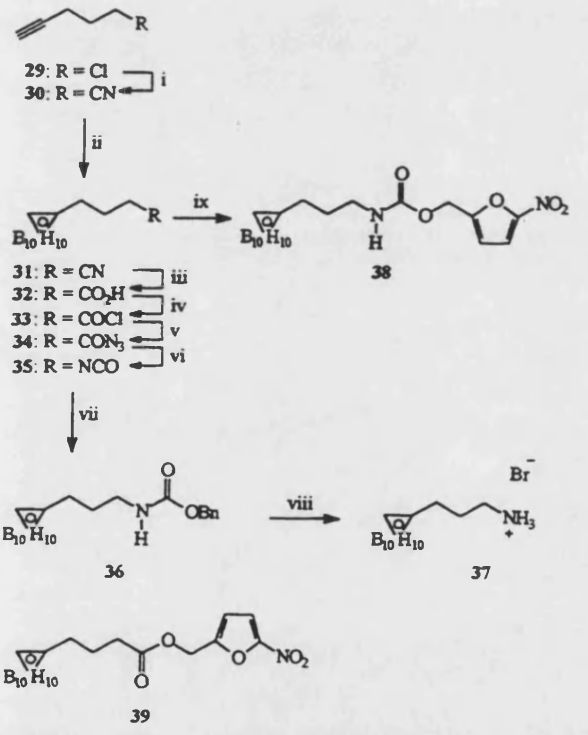
provides another target compound in which the biomimetic reductively triggered release from a pro-drug **4** can be studied. 3-Phenylpropanoic acid **23** was converted into its acid chloride **24** and hence to the acid azide **25** (Scheme 5). Curtius



Scheme 5 Synthesis of nitrofuranylmethyl *N*-(2-phenylethyl)carbamate **27**. *Reagents:* i, $(COCl)_2$; ii, Me_3SiN_3, PhMe; iii, heat, PhMe; iv, **7a**.

rearrangement in boiling toluene gave the isocyanate **26** which was not isolated but was trapped by reaction with **7a** under basic conditions, giving the nitrofuranylmethyl carbamate **27**. From some runs of this reaction, significant yields of the ester **28** were also isolated, indicating incomplete Curtius rearrangement.

Scheme 6 shows the application of this sequence to the synthesis of the nitrofuranylmethyl *N*-(carboranylalkyl)carbamate **38**. Although the carboranebutanoic acid **32** has been reported[30] to be formed by carboxylation of the Grignard reagent derived from 1-(3-bromopropyl)-1,2-dicarba-*closo*-dodecaborane(12), the yield is low, owing to a competing cyclisation to give cyclopentano[1,2]-1,2-dicarba-*closo*-dodecaborane(12). An alternative method, in which all the



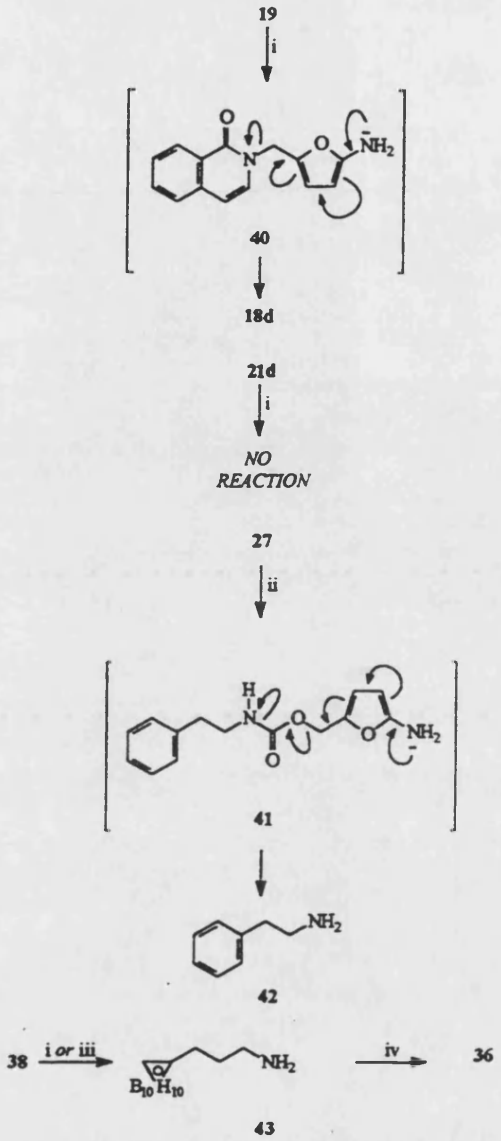
Scheme 6 Synthesis of nitrofuranylmethyl *N*-(3-carboranylpropyl)-carbamate **38**. *Reagents:* i, KCN, EtOH, water; ii, $B_{10}H_{14}$, MeCN; iii, H_2SO_4, water; iv, $SOCl_2$, DMF; v, NaN_3, Me_2CO, water; vi, heat, $CHCl_3$; vii, BnOH, Et_3N, $CHCl_3$; viii, HBr, HOAc; ix, **7a**, Et_3N, $CHCl_3$.

required carbon atoms were present as the carborane was formed, was therefore developed. Hex-5-ynenitrile **30** was prepared straightforwardly from 5-chloropentyne **29**. Following the standard method for synthesis of carboranes from alkynes and decaborane(14) at elevated temperature in the presence of a Lewis base,[16,17,31] the cyanopropylcarborane **31** was prepared in excellent yield. Acidic hydrolysis afforded the carboranebutanoic acid **32**. Formation of the acid chloride **33**, substitution with sodium azide and Curtius rearrangement of **34** in warm chloroform afforded the isocyanate **35**. This was not isolated but was treated with benzyl alcohol under basic conditions to give the Z-protected carboranylpropylamine **36**. This sequence served both as a model for the reaction of **35** with arylmethanols and as an entry into the synthesis of the hitherto unreported carboranylpropylamine. Interestingly, treatment of **36** with hydrogen in the presence of palladium did not effect deprotection and it was necessary to remove the Z group with hydrogen bromide to give the salt **37**. With the carboranylpropylamine 'drug' **37** now available, the corresponding pro-drug **38** was prepared by addition of nitrofuranylmethanol **7a** to the isocyanate **35**. As with the phenylethyl series above, quantities of the analogous nitrofuranylmethyl ester **39** were obtained from some runs, again indicating incomplete Curtius rearrangement.

A mild method for selective chemical reduction of the nitro group was needed to test release of 'drugs' from the two types of pro-drug **1** and **4**. In particular, the conditions must not permit hydrogenolysis of the 'benzylic' CH_2–O or CH_2–N bonds, which would not be biomimetic for the nitroreductases and the cytochrome P450 reductase enzymes. Sodium borohydride in the presence of palladium fulfils these criteria,[17,32] although the usual solvent, methanol, was replaced by propan-2-ol in these studies to minimise any alcoholysis. Firstly, isoquinolinone **18d** was released in 67% yield by this method from pro-drug **19**, through the intermediacy of the aminofuran **40** (Scheme 7). The failure of the furanylmethyl analogue **21d** to release **18d** under the same conditions serves to validate the selectivity of the reduction by excluding a benzylic hydrogenolysis mechanism. An analogous selective reduction of the nitro group in the nitrofuranylmethyl carbamate **27** caused release of 2-phenylethylamine **42** in satisfactory yield, *via* the aminofuran **41**. The physical properties of the carboranylpropylamine were not conducive to easy isolation from boron-containing by-products in this reaction mixture. Therefore, after reductively triggered cleavage, the amine **43** was trapped as its Z derivative **36** by treatment with benzyl chloroformate. The Z-trapped drug was obtained in 26% yield, the relatively low yield being probably due to the isolation procedure. As a final positive control experiment, the pro-drug **38** was subjected to selective reduction of the nitro group by tin(II) chloride.[33] After the tin complex had been decomposed with sodium hydroxide, the carboranylpropylamine **43** was isolated in 40% yield.

A preliminary evaluation of the biological activities of the pro-drug **19** and the corresponding delivered drug **18d** was made to check that the pro-drug is indeed a less potent inhibitor of the target enzyme, PARP, than is the 2-unsubstituted isoquinolin-1-one drug. PARP was extracted with aqueous sodium chloride (0.4 M) from nuclei isolated from L929 murine areolar cells. The enzyme activity was measured in the presence and absence of test compounds by the rate of incorporation of radioactivity from NAD^+ labelled with ^3H in the adenosine into acid-insoluble material. At the test concentration, 10 μM, the pro-drug **19** inhibited the enzyme by 60% whereas isoquinolin-1-one **18d** inhibited the activity by >95% at the same concentration.

In conclusion, it can be seen that a potential bioreductively triggered pro-drug system has been developed, based on reduction of 5-nitrofuran-2-ylmethyl derivatives. The nitrofuranylmethyl group has been linked directly to the 2-position of iso-



Scheme 7 Reductively activated release of isoquinolinone **18d** and amines **42** and **43** from nitrofuranylmethyl ether **19** and carbamates **27** and **38**, respectively. *Reagents:* i, $NaBH_4$, Pd–C, Pr^iOH, water; ii, $NaBH_4$, Pd–C, MeOH, water; iii, $SnCl_2$, HCl, water; iv, ZCl, Et_3N, 4-pyrrolidinylopyridine, CH_2Cl_2.

quinolinone and through a carbamate to a carboranylalkylamine. Efficient syntheses of isoquinolinones **18a–c** and arylmethylations of these isoquinolinones have been developed. New selective methods for nitration of a furan in the competing presence of an isoquinolinone have been identified. Biomimetic reduction of the nitro group initiated efficient expulsion of isoquinolinone **18d** from pro-drug **19** and of the carboranylpropylamine **43** and carbon dioxide from the nitrofuranylmethyl carbamate pro-drug **38**. This pro-drug strategy could be extended into use of nitroheterocycles of different redox potentials carrying other functionalities to modify the physical properties and biodistribution of the pro-drugs.

Experimental

NMR Spectra were obtained of solutions in deuteriochloroform, unless otherwise stated; *J* values are given in Hz. Solutions in organic solvents were dried with anhydrous magnesium sulfate, unless otherwise noted. Solvents were evaporated under reduced pressure. The stationary phase for chromatography was silica gel. Brine refers to saturated aqueous sodium chloride. Ether refers to diethyl ether, unless otherwise stated. DMF refers to dry dimethylformamide, THF refers to dry tetrahydrofuran and EtOAc refers to ethyl acetate.

5-Nitrofuran-2-ylmethanol 7a

5-Nitrofuran-2-carbaldehyde **6** (3.8 g, 27 mmol) was boiled under reflux with aluminium isopropoxide (5.5 g, 27 mmol) in propan-2-ol (50 cm^3) for 4 h and the solvent was then evaporated. After the mixture had been treated with hydrochloric acid (1 M; 50 cm^3) and diluted with ether, it was washed (water), dried and evaporated. Chromatography (EtOAc–hexane, 1:1) of the residue gave the alcohol **7a** (3.8 g, 99%) as a pale yellow oil (lit.,[18,34] oil); δ_H 2.72 (1 H, br s, OH), 4.73 (2 H, s, CH_2), 6.57 (1 H, d, *J* 3.7, furan 3-H) and 7.30 (1 H, d, *J* 3.7, furan 4-H).

2-Chloromethyl-5-nitrofuran 8a

Thionyl chloride (383 mg, 2.8 mmol) in chloroform (1.2 cm^3) was added during 5 min to **7a** (220 mg, 1.5 mmol) in chloroform (1.2 cm^3) and pyridine (0.30 cm^3) at −10 °C and the mixture was stirred at −10 °C for 3 h. It was then washed twice with hydrochloric acid (1 M) and once with aqueous sodium hydroxide (3%), dried and evaporated. Chromatography (EtOAc–hexane, 1:1) of the residue gave the chloromethylnitrofuran **8a** (40 mg, 16%) as a yellow oil (lit.,[35,36] oil); δ_H 4.61 (2 H, s, CH_2), 6.64 (1 H, d, *J* 3.7, furan 3-H) and 7.29 (1 H, d, *J* 3.7, furan 4-H); *m/z* (EI) 163/161 (M) and 126 (M − Cl).

2-(4-Methylphenylsulfonyloxymethyl)-5-nitrofuran 9

A mixture of 4-methylbenzenesulfonyl chloride (2.7 g, 14 mmol), **7a** (2.0 g, 14 mmol) and potassium hydroxide powder (2.0 g, 35 mmol) in THF (75 cm^3) was stirred for 4 h after which it was evaporated. The residue was dissolved in EtOAc, and the solution was washed twice with water, dried and evaporated. Chromatography (dichloromethane–hexane, 1:1) of the residue gave the tosylate **9** (1.06 g, 25%) as a yellow wax (compound reported by Adams *et al.*[37]); δ_H 2.45 (3 H, s, Me), 5.10 (2 H, s, CH_2), 6.64 (1 H, d, *J* 3.7, furan 3-H), 7.20 (1 H, d, *J* 3.7, furan 4-H), 7.35 (2 H, d, *J* 8.0, Ar 3,5-H_2) and 7.78 (2 H, d, *J* 8.0, Ar 2,6-H_2); *m/z* (CI) 298 (M + H).

2-Iodobenzaldehyde 11a

2-Iodophenylmethanol **10** (20.0 g, 85.5 mmol) was stirred with pyridinium dichromate (49.2 g, 130 mmol) in dichloromethane (195 cm^3) for 3 h, after which the mixture was diluted with ether (60 cm^3), filtered and distilled to give the aldehyde **11a** (12.33 g, 62%) as a pale yellow wax, bp 109 °C/1.5 mmHg (lit.,[38] bp 129 °C/1.5 mmHg, lit.,[39] mp 37 °C); δ_H 7.29 (1 H, dt, *J* 1.8, 7.7, 5-H), 7.46 (1 H, t, *J* 7.7, 4-H), 7.88 (1 H, dd, *J* 7.7, 1.8, 3-H), 7.96 (1 H, d, *J* 7.7, 6-H) and 10.08 (1 H, s, CHO).

2-(Diethoxymethyl)methylbenzene 12

Thionyl chloride (5 cm^3) was added to dry ethanol (50 cm^3) at 5 °C, followed by **11b** (10.0 g, 83 mmol) and triethyl orthoformate (100 g, 676 mmol). The mixture was boiled under reflux for 5 h, after which it was cooled and treated with sodium carbonate (10 g). After 15 min the mixture was diluted with ether (150 cm^3) and filtered. After evaporation of the filtrate, the residue was dissolved in EtOAc, washed with aqueous potassium metabisulfite (10%) and water, dried and evaporated to give the acetal **12** (12.3 g, 76%) as a colourless oil (lit.,[40] oil); δ_H 1.23 (6 H, t, *J* 7.0, 2 × CH_2CH_3), 3.53 (2 H, dq, *J* 9.3, 7.0, CH_2CH_3), 3.60 (2 H, dq, *J* 9.3, 7.0, CH_2CH_3), 5.56 [1 H, s, $CH(OEt)_2$], 7.15–7.25 (3 H, m, Ar 3,4,5-H_3) and 7.56 (1 H, m, Ar 6-H). This compound was taken forward without further characterisation.

(*E*)-3-(2-Iodophenyl)propenoic acid 13a

2-Iodobenzaldehyde **11a** (5.00 g, 21.5 mmol) was boiled under reflux with propanedioic acid (4.89 g, 47 mmol) and piperidine (0.2 cm^3) in pyridine (10 cm^3) for 1 h. The cooled mixture was

added to hydrochloric acid (2 M; 150 cm^3) to give a precipitate. This was washed with water and dried to give the acid **13a** (4.86 g, 82%) as colourless needles, mp 218–220 °C (lit.,[41] mp 212–214 °C); δ_H 6.48 (1 H, d, *J* 15.8, 2-H), 7.16 (1 H, t, *J* 8.1, Ar 4-H), 7.44 (1 H, t, *J* 8.1, Ar 5-H), 7.63 (1 H, d, *J* 15.8, 3-H), 7.83 (2 H, m, Ar 3,6-H_2) and 12.67 (1 H, br, CO_2H).

(*E*)-3-(2-Methylphenyl)propenoic acid 13b

The acetal **12** was treated with propanedioic acid, pyridine and piperidine as for the synthesis of **13a** from **11a**, except that the reaction time was 3.5 h. Chromatography (EtOAc–hexane, 1 : 1) of the crude product gave the acid **13b** (3.62 g, 86%) as a white solid, mp 178 °C (lit.,[24] 180 °C); δ_H 2.47 (3 H, s, Me), 6.39 (1 H, d, *J* 15.9, 2-H), 7.20–7.35 (3 H, m, Ar 3,4,5-H_3), 7.59 (1 H, *ca.* d, *J ca.* 7.5, Ar 6-H), 8.10 (1 H, d, *J* 15.9, 3-H) and 11.5 (1 H, br, CO_2H).

(*E*)-3-(2-Bromophenyl)propenoic acid 13c

2-Bromoiodobenzene **14** (14.05 g, 50 mmol) was boiled under reflux with propenoic acid (4.73 g, 66 mmol), palladium(II) acetate (111 mg, 490 μmol) and triethylamine (12.55 g, 124 mmol) in propanenitrile (20 cm^3) for 1.5 h. Hydrochloric acid (2 M; 800 cm^3) was added to the cooled mixture. A solution of the resulting precipitate, in hot ethanol, was filtered and cooled to give the acid **13c** (8.53 g, 76%) as white crystals, mp 202–204 °C (decomp.) (lit.,[42] mp 212–212.5 °C); δ_H[$(CD_3)_2SO$] 6.57 (1 H, d, *J* 16.1, 2-H), 7.35 (1 H, dt, *J* 1.5, 7.7, Ar 4-H), 7.44 (1 H, t, *J* 7.7, Ar 5-H), 7.71 (1 H, dd, *J* 7.7, 1.5, Ar 6-H), 7.83 (1 H, d, *J* 16.1, 3-H), 7.90 (1 H, dd, *J* 7.7, 1.5, Ar 3-H) and 12.66 (1 H, br, CO_2H).

5-Iodoisoquinolin-1-one 18a

The acid **13a** (4.00 g, 14.6 mmol) was stirred with thionyl chloride (10 cm^3) and DMF (0.05 cm^3) for 16 h and then evaporated. The residue (crude **15a**), in 1,4-dioxane (5 cm^3), was added to sodium azide (2.85 g, 44 mmol) in water (6 cm^3) and 1,4-dioxane (6 cm^3) during 15 min. After the mixture had been stirred for 45 min, it was diluted with water (11 cm^3) and extracted thrice with dichloromethane. The dried extract was evaporated to give a residue (crude **16a**) which, in dichloromethane (10 cm^3), was added to boiling dry bis[2-(2-methoxyethoxy)ethyl] ether (12 cm^3) in portions. The solution was boiled under reflux for 1 h and then cooled. The resulting solid was recrystallised (acetone) to give *5-iodoisoquinolin-1-one* **18a** (1.65 mg, 42%) as white needles, mp 238–244 °C (decomp.) (Found: C, 39.8; H, 2.32; N, 5.06. C_9H_6INO requires C, 39.9; H, 2.23; N, 5.17%); δ_H[$(CD_3)_2SO$] 6.55 (1 H, d, *J* 7.3, 4-H), 7.23 (1 H, t, *J* 7.7, 7-H), 7.31 (1 H, d, *J* 7.3, 3-H), 8.22 (2 H, m, 6,8-H_2) and 11.52 (1 H, br, NH); *m/z* (EI) 270.9492 (M, 100%. C_9H_6INO requires *M*, 270.9494).

5-Methylisoquinolin-1-one 18b

Compound **13b** (1.4 g, 8.6 mmol) was stirred with thionyl chloride (15 cm^3) and DMF (0.05 cm^3) for 16 h and then evaporated. The residue (crude **15b**) was stirred with sodium azide (2.0 g, 30 mmol) in acetone (14 cm^3), water (4 cm^3) and acetone (4 cm^3) for 50 min at 5 °C after which it was treated with diphenyl ether (10 cm^3). The suspension was washed with water, dried ($CaCl_2$) and evaporated. The residual solution of crude **16b** was added to boiling diphenyl ether (15 cm^3) during 15 min and the mixture was heated at reflux for 2 h. It was then evaporated and the residue was chromatographed (EtOAc) to give the isoquinolinone **18b** (900 mg, 66%) as a white solid, mp 180–181 °C (lit.,[43] mp 182–183 °C); δ_H 2.55 (3 H, s, Me), 6.71 (1 H, d, *J* 7.3, 4-H), 7.25 (1 H, d, *J* 7.3, 3-H), 7.40 (1 H, dd, *J* 7.9, 7.0, 7-H), 7.51 (1 H, d, *J* 7.0, 6-H), 8.31 (1 H, d, *J* 7.9, 8-H) and 12.16 (1 H, br, NH); δ_C 19.17 (CH_3), 103.47 (CH), 125.18 (CH), 125.95 (C_q), 126.10 (C_q), 126.39 (CH), 127.47 (CH), 133.45 (CH), 137.18 (C_q) and 164.90 (C_q); *m/z* (EI) 160.0712 (M. $C_9{}^{13}CH_9NO$ requires *M*, 160.0718) and 159.0682 (100%, M. $C_{10}H_9NO$ requires *M*, 159.0684).

5-Bromoisoquinolin-1-one 18c

Triethylamine (1.6 g, 16 mmol) was added to **13c** (3.00 g, 13 mmol) in dry acetone (35 cm^3) at 0 °C followed by ethyl chloroformate (1.72 g, 16 mmol) in dry acetone (3 cm^3). The mixture was stirred at 0 °C for 30 min, after which it was treated with sodium azide (1.2 g, 18.5 mmol) in water (3 cm^3), added during 15 min. The mixture was poured onto ice and extracted with dichloromethane. The extract was dried ($CaCl_2$) and then added carefully to boiling diphenyl ether (10 cm^3). The solution was boiled under reflux for 1 h after which it was evaporated and the residue was chromatographed (ether). Recrystallisation (acetonitrile) of the crude product gave the *title compound* **18c** (305 mg, 10%) as white needles, mp 242–244 °C (Found: C, 47.9; H, 2.59; N, 6.14. C_9H_6BrNO requires C, 48.2; H, 2.70; N, 6.25%); δ_H[$(CD_3)_2SO$] 6.65 (1 H, d, *J* 7.3, 4-H), 7.36 (1 H, t, *J* 7, 7-H), 7.42 (1 H, d, *J* 7.3, 3-H), 8.02 (1 H, d, *J* 7, 6-H), 8.21 (1 H, d, *J* 7, 8-H) and 11.55 (1 H, br, NH); *m/z* (EI) 224.9628 (M. $C_9H_6{}^{81}BrNO$ requires *M*, 224.9612) and 222.9635 (M. $C_9H_6{}^{79}BrNO$ requires *M*, 222.9633).

2-(5-Nitrofuran-2-ylmethyl)isoquinolin-1-one 19

Method A. Sodium hydride (60% in oil; 6.0 mg, 153 μmol) was stirred with **18d** (22 mg, 153 μmol) in DMF (1.0 cm^3) for 1 h and the mixture was then cooled to 5 °C. Compound **9** (50 mg, 168 μmol) was added to the mixture which was then stirred for 2 d at 20 °C. Evaporation of the mixture and chromatography (EtOAc) of the residue gave the *title compound* **19** (10 mg, 24%), the properties of which are described below.

Method B. Conc. nitric acid (60%; 0.10 cm^3) was stirred with **21d** (118 mg, 520 μmol) in trifluoroacetic acid (1.0 cm^3) at −10 °C for 1 h and at 20 °C for 16 h. The mixture was adjusted to pH 5 with aqueous sodium hydroxide (2 M) and was then extracted with EtOAc. The extract was washed with water, aqueous sodium hydrogen carbonate and brine, dried and evaporated. Chromatography (first column using EtOAc–hexane, 1 : 1; and then a second column using dichloromethane) of the residue afforded the *nitrofuran* **19** (21 mg, 17%) as a yellow solid, mp 77–79 °C; δ_H 5.23 (2 H, s, CH_2), 6.58 (1 H, d, *J* 7.3, isoquinoline 4-H), 6.66 (1 H, d, *J* 3.6, furan 3-H), 7.24 (1 H, d, *J* 7.3, isoquinoline 3-H), 7.25 (1 H, d, *J* 3.6, furan 4-H), 7.5 (2 H, m, isoquinoline 5,7-H_2), 7.57 (1 H, br t, *J* 7.5, isoquinoline 6-H) and 8.39 (1 H, br d, *J* 7.9, isoquinoline 8-H); *m/z* (EI) 270.0637 (M. $C_{14}H_{10}N_2O_4$ requires *M*, 270.0641) and 224 (M − NO_2).

Method C. Copper(II) nitrate (118 mg, 480 μmol) was added to **21d** (55 mg, 240 μmol) in trifluoroacetic acid (2.0 cm^3) at −20 °C and the mixture was stirred at 20 °C for 2 d. Work-up as for Method B afforded the *nitrofuran* **19** (28 mg, 42%) with properties as described above.

5-Iodo-2-phenylmethylisoquinolin-1-one 20a

Lithium hexamethyldisilazide (1.0 M in THF; 0.6 cm^3, 600 μmol) was stirred with **18a** (100 mg, 370 μmol) in THF (10 cm^3) for 2 h, after which chloromethylbenzene (60 mg, 480 μmol) in THF (10 cm^3) was added to the mixture, followed by sodium iodide (5 mg). After the mixture had been stirred for 5 d, it was evaporated and the residue, in EtOAc, was washed with water and brine, dried and evaporated. Chromatography (EtOAc–hexane, 3 : 7) of the residue gave the *N-benzylisoquinolinone* **20a** (123 mg, 92%) as a white solid, mp 118–120 °C (Found: H, 3.46; N, 3.82. $C_{16}H_{12}INO$ requires H, 3.35; N, 3.88%); ν_{max}(KBr disc)/cm^{-1} 1650, 1620 and 1585; δ_H 5.22 (2 H, s, CH_2), 6.72 (1 H, d, *J* 7.3, isoquinoline 4-H), 7.19 (2 H, m, isoquinoline 3,7-H_2), 7.33 (5 H, m, Ph-H_5), 8.15 (1 H, dd, *J* 7.7, 1.3, isoquinoline 6-H) and 8.47 (1 H, dd, *J* 8.1, 1.3, isoquinoline 8-H); δ_C 51.90 (CH_2), 96.29 (C_q), 109.91 (CH), 127.21 (C_q), 127.96 (CH), 128.14 (CH + C_q), 128.69 (CH), 128.86 (CH), 132.55 (CH), 136.41

(C_q), 139.02 (C_q), 143.01 (CH) and 161.45 (C_q); *m/z* (EI) 360.9960 (M. $C_{16}H_{12}INO$ requires *M*, 360.9964) and 91 (100%, Bn).

5-Methyl-2-phenylmethylisoquinolin-1-one 20b

Sodium hydride (60% in oil; 13 mg, 320 μmol) was stirred with **18b** (50 mg, 314 μmol) in DMF (3.0 cm^3) for 1 h after which bromomethylbenzene (59 mg, 350 μmol) was added to the mixture; stirring was then continued for 20 h. Evaporation of the mixture gave a residue, which, as a solution in EtOAc, was washed with water, dried and evaporated. Chromatography (dichloromethane→dichloromethane–methanol 49:1) of the residue furnished the N-*benzylisoquinolinone* **20b** (15 mg, 19%) as a white solid, mp 84–86 °C; δ_H 2.51 (3 H, s, Me), 5.23 (2 H, s, CH_2), 6.61 (1 H, d, *J* 7.6, isoquinoline 4-H), 7.12 (1 H, d, *J* 7.6, isoquinoline 3-H), 7.30–7.35 (5 H, m, Ph-H_5), 7.38 (1 H, t, *J* 7.6, isoquinoline 7-H), 7.47 (1 H, br d, *J* 7.5, isoquinoline 6-H) and 8.34 (1 H, br d, *J* 7.5, isoquinoline 8-H); δ_C 18.93 (CH_3), 51.68 (CH_2), 103.13 (CH), 126.07 (CH), 126.58 (C_q), 127.80 (CH), 127.93 (CH), 128.39 (C_q), 128.79 (CH), 130.90 (CH), 133.03 (CH), 133.16 (CH), 135.91 (C_q), 136.91 (C_q) and 162.50 (C_q); *m/z* (EI) 249.1153 (M. $C_{17}H_{15}NO$ requires *M*, 249.1154) and 91 (100%, Bn).

5-Bromo-2-(4-methoxyphenylmethyl)isoquinolin-1-one 20c

Compound **18c** was treated with lithium hexamethyldisilazide, sodium iodide and 1-chloromethyl-4-methoxybenzene in THF as for the synthesis of **20a** to give the *title compound* **20c** (156 mg, 100%) as a white solid, mp 98–100 °C (Found: C, 59.4; H, 4.18; N, 3.88. $C_{17}H_{14}BrNO$ requires C, 59.3; H, 4.10; N, 4.07%); δ_H 3.78 (3 H, s, Me), 5.15 (2 H, s, CH_2), 6.84 (3 H, m, isoquinoline 4-H + Ar 3,5-H_2), 7.18 (1 H, d, *J* 7.7, isoquinoline 3-H), 7.3 (3 H, m, isoquinoline 7-H + Ar 2,6-H_2), 7.87 (1 H, d, *J* 7.7, isoquinoline 6-H) and 8.43 (1 H, d, *J* 8, isoquinoline 8-H); *m/z* (CI) 345/343 (M + H) and 121 (100%, MeOBn).

2-Phenylmethylisoquinolin-1-one 20d

Sodium hydride (60% in oil; 15 mg, 340 μmol) was stirred with **18d** (50 mg, 340 μmol) in DMF (3.0 cm^3) for 1 h after which bromomethylbenzene (65 mg, 380 μmol) was added to the mixture; stirring was continued for 2 h. Evaporation of the mixture gave a residue which, as a solution in EtOAc, was washed with water, dried and evaporated. Chromatography (dichloromethane–methanol, 19:1) of the residue gave the *title compound* **20d** (77 mg, 96%) as a colourless oil (lit.,[44] mp 67–69 °C); δ_H 5.20 (2 H, s, CH_2), 6.46 (1 H, d, *J* 7.5, isoquinoline 4-H), 7.06 (1 H, d, *J* 7.4, isoquinoline 3-H), 7.30 (5 H, m, Ph-H_5), 7.49 (2 H, m, isoquinoline 5,7-H_2), 7.61 (1 H, dt, *J* 1.5, 7.5, isoquinoline 6-H) and 8.42 (1 H, br d, *J* 7.5, isoquinoline 8-H); δ_C 51.59 (CH_2), 106.37 (CH), 125.85 (CH), 126.20 (C_q), 126.80 (CH), 127.73 (CH), 127.86 (CH), 127.96 (CH), 128.70 (CH), 131.20 (CH), 132.14 (CH), 136.81 (C_q), 136.91 (C_q) and 162.16 (C_q); *m/z* (EI) 236.1031 (M. $C_{15}{}^{13}CH_{13}NO$ requires *M*, 236.1031) and 235.0995 (M. $C_{16}H_{13}NO$ requires *M*, 235.0997); *m/z* (FAB positive ion) 236 (100%, M + H) and 91 (Bn).

2-(Furan-2-ylmethyl)-5-methylisoquinolin-1-one 21b

Lithium hexamethyldisilazide (1.0 M in THF; 6.0 cm^3, 6.0 mmol) was stirred with **18b** (450 mg, 2.8 mmol) in THF (40 cm^3) for 2 h. Crude **8b** (as in the synthesis of **21d**) (3.3 g, 28 mmol) in THF (30 cm^3) was added to this solution at 0 °C and the mixture was boiled under reflux for 3 d. Evaporation of the mixture gave a residue which, dissolved in EtOAc, was washed with water and brine, dried and evaporated. Chromatography (EtOAc–hexane, 1:1) and further chromatography (EtOAc–hexane, 1:5) of the residue gave the *title compound* **21b** (84 mg, 12%) as a pale yellow solid, mp 85–87 °C (lit.,[45] mp 84–86 °C for 2-([^{18}O]furan-2-ylmethyl)-5-methylisoquinolin-1-one (8% isotopic enrichment); δ_H 2.51 (3 H, s, Me), 5.20 (2 H, s, CH_2), 6.34 (1 H, dd, *J* 3.1, 1.8, furan 4-H), 6.42 (1 H, d, *J* 3.1, furan 3-H), 6.62 (1 H, d, *J* 7.5, isoquinoline 4-H), 7.21 (1 H, d, *J* 7.5, isoquinoline 3-H), 7.37 (2 H, m, furan 5-H + isoquinoline 7-H), 7.46 (1 H, br d, *J* 7.2, isoquinoline 6-H) and 8.32 (1 H, br d, *J* 8.0, isoquinoline 8-H); δ_C 18.91 (CH_3), 44.33 (CH_2), 103.04 (CH), 109.42 (CH), 110.64 (CH), 125.95 (CH), 126.37 (C_q), 126.54 (CH), 130.54 (CH), 133.06 (CH), 133.17 (CH), 135.90 (C_q), 142.76 (CH), 149.78 (C_q) and 162.14 (C_q); *m/z* (EI) 240.0980 (M. $C_{14}{}^{13}CH_{13}NO_2$ requires *M*, 240.0980), 239.0945 (M. $C_{15}H_{13}NO_2$ requires *M*, 239.0946) and 81 (furanCH_2).

2-(Furan-2-ylmethyl)isoquinolin-1-one 21d

Thionyl chloride (4.05 g, 35 mmol) in chloroform (5 cm^3) was added during 10 min to **7b** (2.0 g, 2 mmol) in chloroform (5 cm^3) and pyridine (3 cm^3) at −10 °C. The mixture was stirred at this temperature for 3 h and then poured into hydrochloric acid (1 M) at 0 °C. The organic phase was separated, washed rapidly with cold hydrochloric acid (1 M) and cold aqueous sodium hydroxide (3%), dried (K_2CO_3) and was evaporated to give crude 2-chloromethylfuran **8b** (1.08 g, 45%) as an unstable pale yellow oil; δ_H 4.63 (2 H, s, CH_2), 6.4 (2 H, m, furan 3,4-H_2) and 7.46 (1 H, dd, *J* 1.8, 0.9, furan 5-H). Lithium hexamethyldisilazide (1.0 M in THF; 7.0 cm^3, 7.0 mmol) was stirred with **18d** (500 mg, 3.4 mmol) in THF (50 cm^3) for 1.5 h after which crude **8b** (1.08 g, 9.3 mmol) in THF (50 cm^3) was added to it at −10 °C, followed by sodium iodide (50 mg). The mixture was stirred at 20 °C for 24 h after which it was evaporated to give a residue, which, dissolved in EtOAc, was washed with water and brine, dried and evaporated. Chromatography (EtOAc–hexane, 1:2) gave the *title compound* **21d** (646 mg, 83%) as a pale yellow oil; δ_H 5.17 (2 H, s, CH_2), 6.31 (1 H, dd, *J* 3.0, 2.0, furan 4-H), 6.40 (1 H, br d, *J* 3.0, furan 3-H), 6.45 (1 H, d, *J* 7.3, isoquinoline 4-H), 7.14 (1 H, d, *J* 7.3, isoquinoline 3-H), 7.35 (1 H, dd, *J* 2.0, 1.0, furan 5-H), 7.44 (1 H, dt, *J* 1.0, 6.8, isoquinoline 7-H), 7.45 (1 H, d, *J* 7.8, isoquinoline 5-H), 7.57 (1 H, dt, *J* 1.0, 7, isoquinoline 6-H) and 8.42 (1 H, br d, *J* 7.8, isoquinoline 8-H); δ_C 44.16 (CH_2), 106.19 (CH), 109.28 (CH), 110.49 (CH), 125.77 (CH), 125.99 (C_q), 126.71 (CH), 127.77 (CH), 130.79 (CH), 132.10 (CH), 136.86 (C_q), 142.62 (CH), 149.62 (C_q) and 161.75 (C_q); *m/z* (EI) 226.0819 (M. $C_{13}{}^{13}CH_{11}NO_2$ requires *M*, 226.0823), 225.0788 (M. $C_{14}H_{11}NO_2$ requires *M*, 225.0790) and 81 (furanCH_2).

4-Nitro-2-(5-nitrofuran-2-ylmethyl)isoquinolin-1-one 22

Fuming nitric acid (90%; 0.09 cm^3, 2.0 mmol) was added to acetic anhydride (0.2 cm^3) at −30 °C. The mixture was stirred with **21d** (92 mg, 410 μmol) in acetic anhydride (1.0 cm^3) at −10 °C for 1 h and then poured onto ice. After adjustment to pH 5 with aqueous sodium hydroxide (2 M), the mixture was extracted with EtOAc. The extract was washed with aqueous sodium hydrogen carbonate and brine, dried and evaporated. Chromatography (EtOAc–hexane, 1:1) of the residue gave the *dinitro compound* **22** (26 mg, 20%) as a yellow oil; δ_H 5.34 (2 H, s, CH_2), 6.78 (1 H, d, *J* 3.8, furan 3-H), 7.29 (1 H, d, *J* 3.7, furan 4-H), 7.66 (1 H, *ca.* t, *J ca.* 8, isoquinoline 6-H or 7-H), 7.88 (1 H, *ca.* t, *J ca.* 8, isoquinoline 7-H or 6-H), 8.46 (1 H, dd, *J* 8.1, 1.2, isoquinoline 5-H or 8-H), 8.68 (1 H, br d, *J* 8.5, isoquinoline 8-H or 5-H) and 8.77 (1 H, s, isoquinoline 3-H); δ_C 45.94 (CH_2), 112.12 (CH), 113.62 (CH), 123.99 (CH), 124.30 (C_q), 128.93 (CH), 129.02 (CH), 129.15 (C_q), 134.75 (CH), 135.00 (C_q), 135.95 (CH), 150.88 (2 × C_q) and 161.08 (C_q); *m/z* (EI) 315.0487 (M. $C_{14}H_9N_3O_6$ requires *M*, 315.0491), 270 (M − NO_2) and 190 (4-nitroisoquinolin-1-one).

5-Nitrofuran-2-ylmethyl *N*-(2-phenylethyl)carbamate 27 and 5-nitrofuran-2-ylmethyl 3-phenylpropanoate 28

3-Phenylpropanoic acid **23** (100 mg, 670 μmol) was stirred with oxalyl chloride (1.0 cm^3) for 2 h after which it was evaporated. The resulting residue (crude **24**) was boiled under reflux with toluene (1 cm^3) and azidotrimethylsilane (87 mg, 730 μmol) for 24 h after which compound **7a** (95 mg, 670 mmol), in toluene (1

cm^3), was added to it; boiling was continued for 4 h. After evaporation of the mixture, the residue, dissolved in EtOAc, was washed with water and brine and evaporated. Chromatography (dichloromethane–hexane, 1:1) gave the *ester* **28** (66 mg, 34%) as a yellow oil; δ_H 2.70 (2 H, t, *J* 7.1, CH_2CO), 2.96 (2 H, t, *J* 7.1, $PhCH_2$), 5.09 (2 H, s, OCH_2), 6.54 (1 H, d, *J* 3.7, furan 3-H) and 7.1–7.3 (6 H, m, Ph-H_5 + furan 4-H); *m/z* (CI) 276.0872 (M + H. $C_{14}H_{14}NO_5$ requires *M*H, 276.0872) and 133 (100%, $PhCH_2CH_2CO$). Further elution gave the *carbamate* **27** (62 mg, 32%) as a colourless gum; δ_H 2.82 (2 H, t, *J* 7.0, $PhCH_2$), 3.46 (2 H, q, *J* 7.1, NCH_2), 4.90 (1 H, br, NH), 5.08 (2 H, s, OCH_2), 6.60 (1 H, d, *J* 3.7, furan 3-H) and 7.1–7.3 (6 H, m, Ph-H_5 + furan 4-H); *m/z* (CI) 291 (M + H) and 122 ($PhCH_2CH_2NH_2$); *m/z* (FAB positive ion) 291.1002 (M + H. $C_{14}H_{15}N_2O_5$ requires *M*H, 291.0981).

Hex-5-ynenitrile 30

A mixture of 5-chloropent-1-yne **29** (10.0 g, 97 mmol), potassium cyanide (**CAUTION**) (9.8 g, 150 mmol), ethanol (100 cm^3) and water (30 cm^3) was boiled under reflux for 2 d after which it was diluted with water (50 cm^3) and extracted with ether. The extract was dried and evaporated and the residue was chromatographed (EtOAc–hexane, 1:1→EtOAc) to give the nitrile **30** (3.2 g, 36%) as a colourless oil (lit.,[46] oil, lit.,[47] liquid); ν_{max}/cm^{-1} 3300, 2260 and 2040; δ_H 1.88 (2 H, quintet, *J* 7.0, 3-H_2), 2.09 (1 H, t, *J* 2.6, 6-H), 2.38 (2 H, dt, *J* 2.6, 7.0, 4-H_2) and 2.52 (2 H, t, *J* 7.0, 2-H_2).

1-(3-Cyanopropyl)-1,2-dicarba-*closo*-dodecaborane(12) 31

Decaborane(14) ($B_{10}H_{14}$; 328 mg, 2.7 mmol) was stirred with dry acetonitrile (5.0 cm^3) for 3 h, after which compound **30** (250 mg, 2.7 mmol) was added to the mixture. After being boiled under reflux for 5 d, the mixture was evaporated and the residue was chromatographed (pentane–dichloromethane, 2:1) to give the cyanopropylcarborane **31** (420 mg, 74%) as a colourless gum (lit.,[13] mp 81–82 °C); ν_{max}/cm^{-1} 2560 and 2260; $\delta_H(CDCl_3)$ 1.86 (2 H, m, $CH_2CH_2CH_2$), 2.2 (10 H, br q, J_{BH} *ca.* 150, 10 × BH), 2.35 (2 H, m, carborane-CH_2), 2.39 (2 H, t, *J* 6.7, CH_2CN) and 3.67 (1 H, br, carborane 2-H); $\delta_H[(CD_3)_2CO]$ 1.87 (2 H, m, $CH_2CH_2CH_2$), 2.2 (10 H, br q, J_{BH} *ca.* 150, 10 × BH), 2.50 (2 H, m, carborane-CH_2), 2.49 (2 H, t, *J* 7.0, CH_2CN) and 4.69 (1 H, br, carborane 2-H); $\delta_B[(CD_3)_2CO]$ −14.09 (2 B, J_{BH} 135), −12.80 (4 B, J_{BH} 145), −10.71 (2 B, J_{BH} 149), −7.04 (1 B, J_{BH} 146) and −3.95 (1 B, J_{BH} 146); *m/z* (EI) cluster centred at 211 (M).

1,2-Dicarba-*closo*-dodecaboran(12)-1-ylbutanoic acid 32

Compound **31** (1.54 g, 7.3 mmol) was boiled under reflux with conc. sulfuric acid (35 cm^3) and water (7 cm^3) for 30 h, after which it was diluted with water (200 cm^3). The resulting precipitate, dissolved in dichloromethane, was washed with water and brine, dried and evaporated to give the acid **32** (1.45 g, 87%) as a white solid, mp 155–157 °C (lit.,[48] mp 158–159 °C); δ_H 1.81 (2 H, m, $CH_2CH_2CH_2$), 2.2 (10 H, br q, J_{BH} *ca.* 150, 10 × BH), 2.27 (2 H, m, carborane-CH_2), 2.37 (2 H, t, *J* 7.0, CH_2CO), 3.58 (1 H, br, carborane 2-H) and 10.5 (1 H, br, CO_2H); *m/z* (EI) cluster centred at 230 (M), cluster centred at 213 (M − OH).

Phenylmethyl *N*-[3-(1,2-dicarba-*closo*-dodecaboran-1-yl)propyl]-carbamate 36

The isocyanate **35** (90 mg, 0.4 mmol) (as in the synthesis of **38**) was boiled under reflux with phenylmethanol (42 mg, 0.4 mmol) and triethylamine (5 mg) in chloroform (5 cm^3) for 24 h. The mixture was evaporated and the residue, dissolved in EtOAc, was washed with water and brine, dried and evaporated. Chromatography (dichloromethane–hexane, 1:1) of the residue gave the *carbamate* **36** (62 mg, 48%) as a pale yellow oil; δ_H 1.69 (2 H, m, $CH_2CH_2CH_2$), 2.2 (10 H, br q, J_{BH} *ca.* 150, 10 × BH), 2.22 (2 H, m, carborane-CH_2), 3.17 (2 H, q, *J* 6.6, NCH_2), 3.58 (1 H, br, carborane 2-H), 4.78 (1 H, br, NH), 5.09 (2 H, s, $PhCH_2$) and 7.35 (5 H, s, Ph-H_5); δ_C 29.97 (CH_2), 35.19 (CH_2), 39.96 (CH_2), 61.27 (CH), 66.91 (CH_2), 74.52 (C_q), 128.15 (CH), 128.30 (CH), 128.59 (CH), 136.26 (C_q) and 156.42 (C_q); $\delta_B(^1H$-decoupled) −11.77 (6 B, m), −9.30 (2 B, s), −5.69 (1 B, s) and −2.25 (1 B, s); *m/z* (EI) 337.2822 (M. $C_{13}H_{25}{}^{11}B_{10}NO_2$ requires 337.2816), 336.2844 (M. $C_{13}H_{25}{}^{11}B_9{}^{10}BNO_2$ requires *M*, 336.2852), 335.2874 (M. $C_{13}H_{25}{}^{11}B_8{}^{10}B_2NO_2$ requires *M*, 335.2888), 334.2898 (M. $C_{13}H_{25}{}^{11}B_7{}^{10}B_3NO_2$ requires *M*, 334.2925), 333.2923 (M. $C_{13}H_{25}{}^{11}B_6{}^{10}B_4NO_2$ requires *M*, 333.2961) and 332.2946 (M. $C_{13}H_{25}{}^{11}B_5{}^{10}B_5NO_2$ requires *M*, 332.2997).

1-(3-Aminopropyl)-1,2-dicarba-*closo*-dodecaborane(12) hydrobromide 37

The carbamate **36** (60 mg, 180 μmol) was stirred with hydrogen bromide in acetic acid (5%; 6 cm^3) for 30 min, after which the mixture was evaporated. The residue was triturated with dry ether (5 × 10 cm^3) and dried to afford the *aminopropylcarborane salt* **37** (39 mg, 98%) as a white solid, mp 295–297 °C; $\delta_H(D_2O)$ 1.86 (2 H, *ca.* quintet, *J ca.* 7, $CH_2CH_2CH_2$), 2.2 (10 H, br q, J_{BH} *ca.* 150, 10 × BH), 2.38 (2 H, *ca.* t, *J ca.* 7, carborane-CH_2), 2.95 (2 H, t, *J* 7.5, NCH_2) and 4.38 (1 H, br, carborane 2-H); *m/z* (EI) 203.2436 (M. $C_5H_{19}{}^{11}B_{10}N$ requires *M*, 203.2448).

5-Nitrofuran-2-ylmethyl *N*-[3-(1,2-dicarba-*closo*-dodecaboran-(12)-1-yl)propyl]carbamate 38 and 5-nitrofuran-2-ylmethyl 4-[1,2-dicarba-*closo*-dodecaboran(12)-1-yl]butanoate 39

Compound **32** (500 mg, 2.2 mmol) was boiled under reflux with thionyl chloride (25 cm^3) and DMF (0.05 cm^3) for 16 h. After the evaporation of the solvent, the residue (crude **33**) was stirred with sodium azide (**CAUTION**) (495 mg, 7.6 mmol) in acetone (25 cm^3) and water (5 cm^3) at 0 °C for 45 min, after which it was diluted with chloroform (100 cm^3). The suspension was washed with water and brine, dried and evaporated to give crude 1,2-dicarba-*closo*-dodecaboran-1-ylbutanoyl azide **34**; ν_{max}/cm^{-1} 2600, 2150 and 1725. This material was stirred in chloroform (10 cm^3) at 40 °C for 27 h to give a solution of crude 1-(3-isocyanatopropyl)-1,2-dicarba-*closo*-carborane(12) **35**; ν_{max}/cm^{-1} 2600 and 2280. This solution was boiled under reflux with **7a** (310 mg, 2.2 mmol) and triethylamine (10 mg) for 2 d after which it was evaporated. The residue, dissolved in EtOAc, was washed with water and brine, dried and evaporated. Chromatography (dichloromethane–methanol, 40:1) of the residue gave the *ester* **39** (90 mg, 14%) as a pale yellow wax; δ_H 1.82 (2 H, m, $CH_2CH_2CH_2$), 2.2 (10 H, br q, J_{BH} *ca.* 150, 10 × BH), 2.25 (2 H, m, carborane-CH_2), 2.38 (2 H, t, *J* 7.0, CH_2CO), 3.60 (1 H, br, carborane 2-H), 5.12 (2 H, s, furan-CH_2), 5.25 (1 H, br, NH), 6.63 (1 H, d, *J* 3.7, furan 3-H) and 7.29 (1 H, d, *J* 3.7, furan 4-H); *m/z* (CI) cluster centred at 355 (M + H), cluster centred at 213 [carborane$(CH_2)_3CO$] and 143 (nitrofuranmethanol); *m/z* (FAB positive ion) 357.2477 (M. $C_{11}H_{22}{}^{11}B_9{}^{10}BNO_5$ requires *M*, 357.2465). Further elution gave the *carbamate* **38** (289 mg, 36%) as a pale yellow oil; δ_H 1.40 (2 H, m, $CH_2CH_2CH_2$), 2.2 (10 H, br q, J_{BH} *ca.* 150, 10 × BH), 2.48 (2 H, m, carborane-CH_2), 3.37 (2 H, *ca.* q, *J ca.* 7, NCH_2), 3.68 (1 H, br, carborane 2-H), 4.73 (2 H, s, furan-CH_2), 5.25 (1 H, br, NH), 6.56 (1 H, d, *J* 3.6, furan 3-H) and 7.29 (1 H, d, *J* 3.6, furan 4-H); δ_C 29.74 (CH_2), 35.13 (CH_2), 40.12 (CH_2), 57.96 (CH_2), 61.35 (CH), 74.39 (C_q), 112.11 (CH), 113.05 (CH), 153.00 (C_q) and 155.43 (C_q) (one C_q was not observed); $\delta_B(^1H$-decoupled) −11.83 (6 B, m), −9.30 (2 B, s), −5.69 (1 B, s) and −2.31 (1 B, s); *m/z* (CI) cluster centred at 371 (M + H); *m/z* (FAB positive ion) 372.2599 (M + H. $C_{11}H_{23}{}^{11}B_9{}^{10}BN_2O_5$ requires MH^+, 372.2574) and 369.2704 (M + H. $C_{11}H_{23}{}^{11}B_6{}^{10}B_4N_2O_5$ requires MH^+, 369.2683).

Reductively activated release of isoquinolin-1-one 18d from 2-(5-nitrofuran-2-ylmethyl)isoquinolin-1-one 19 (sodium borohydride–palladium method)

Sodium borohydride (16 mg, 410 μmol) in water (0.3 cm^3) was

stirred with **19** (37 mg, 140 μmol) and palladium-on-charcoal (10%; 4 mg) in propan-2-ol (2.0 cm^3) for 16 h after which the suspension was filtered through Celite®. Evaporation of the mixture gave a residue, which, dissolved in dichloromethane, was washed with water and brine, dried and evaporated to give isoquinolin-1-one **18d** (13 mg, 67%) with properties as described above.

Control experiment for sodium borohydride–palladium method of reductively activated release

Sodium borohydride (15 mg, 400 μmol) in water (0.3 cm^3) was stirred with **21d** (30 mg, 133 μmol) and palladium-on-charcoal (10%; 3 mg) in propan-2-ol (1.0 cm^3) for 2 d. After the suspension had been filtered through Celite®, it was evaporated and the residue, dissolved in dichloromethane, was washed with water and brine, dried and evaporated to give recovered **21d** (28 mg, 94%).

Reductively activated release of 2-phenylethylamine 42 from 5-nitrofuran-2-ylmethyl *N*-(2-phenylethyl)carbamate 27 (sodium borohydride–palladium method)

Sodium borohydride (18 mg, 470 μmol) in water (0.15 cm^3) was stirred with **27** (30 mg, 100 μmol) and palladium-on-charcoal (10%; 3 mg) in methanol (2.0 cm^3) for 2 d. After the suspension had been filtered through Celite®, it was evaporated and the residue, dissolved in dichloromethane, was washed with water and brine, dried and evaporated to give 2-phenylethylamine **42** (5.0 mg, 41%), the properties of which were identical with those of a commercial sample.

Reductively activated release of 1-(3-aminopropyl)-1,2-dicarba-*closo*-dodecaborane(12) 43 from 5-nitrofuran-2-ylmethyl *N*-{3-[1,2-dicarba-*closo*-dodecaboran(12)-1-yl]propyl}carbamate 38

Sodium borohydride–palladium method. Sodium borohydride (33 mg, 870 μmol) in water (1.0 cm^3) was stirred with **38** (100 mg, 270 μmol) and palladium-on-charcoal (10%; 10 mg) in propan-2-ol (5.0 cm^3) for 16 h. Filtration (Celite®) and evaporation of the mixture gave crude 1-(3-aminopropyl)-1,2-dicarba-*closo*-dodecaborane(12) **43**. This material, dissolved in dichloromethane (10 cm^3), was stirred with phenylmethyl chloroformate (76 mg, 450 μmol), triethylamine (75 mg, 750 μmol) and 4-pyrrolidinylpyridine (2 mg) for 16 h. The solution was washed with water, aqueous citric acid (5%) and brine, dried and evaporated. Chromatography of the residue gave the Z-protected carboranylpropylamine **36** (14 mg, 26%), the properties of which were identical with those reported above.

Tin(II) chloride method. Tin(II) chloride (74 mg, 390 μmol) and **38** (19 mg, 65 μmol) were boiled under reflux in hydrochloric acid (1.5 cm^3) for 90 min. The cooled mixture was basified to pH 9 by the addition of aqueous sodium hydroxide (10 M) and extracted with dichloromethane. The extract was washed with water, dried and evaporated to give the amine **43** (3 mg, 40%) as a pale yellow gum, chromatographically identical with the free base of **37**.

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Labelled Compounds of Interest as Antitumour Agents - V.
Syntheses of [^{18}O]-5-Methylisoquinolinone and
1-(Furan-2-yl-[^{18}O]-methoxy)-5-methylisoquinoline: Correction

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NOTE

LABELLED COMPOUNDS OF INTEREST AS ANTITUMOUR AGENTS - V. SYNTHESES OF [^{18}O]-5-METHYLISOQUINOLINONE AND 1-(FURAN-2-YL-[^{18}O]-METHOXY)-5-METHYLISOQUINOLINE: CORRECTION

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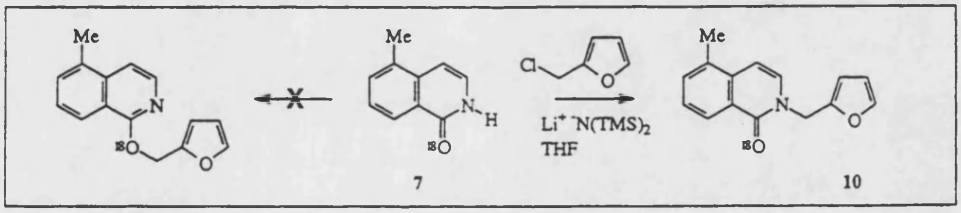
In a recent paper[1] from our laboratory, we reported that the reaction of the lithium anion of 5-methylisoquinolin-[^{18}O]-one with 2-(chloromethyl)furan gave 1-(furan-2-yl-[^{18}O]-methoxy)-5-methylisoquinoline, the product of alkylation at oxygen, in 60% chemical yield. The few literature precedents[2-4] for alkylation of anions derived from isoquinolin-1-ones show that the major or sole products are the corresponding 2-alkylisoquinolin-1-ones derived from alkylation at nitrogen, irrespective of the nature of the electrophile. Re-examination of the spectroscopic data leads us to the conclusion that the product is 2-(furan-2-ylmethyl)-5-methylisoquinolin-[^{18}O]-one, arising from furanylmethylation at nitrogen. In particular, the chemical shift of the CH_2 in the ^{13}C NMR spectrum (δ_C 44.33) is consistent with typical values for $ArCH_2N$ but not $ArCH_2O$.



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Reaction of Hydrazinoquinolines with Trifluoromethyl β-Diketones: Structural and Mechanistic Studies

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Reaction of hydrazinoquinolines with trifluoromethyl-β-diketones: structural and mechanistic studies

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Abstract

Reaction of 2-hydrazino-4-methylquinoline with a series of trifluoromethyl-β-diketones gives 3-substituted-5-hydroxy-1-(4-methylquinolin-2-yl)-5-trifluoromethyl-4,5-dihydropyrazoles and, in some cases, 5-substituted-1-(4-methylquinolin-2-yl)-3-trifluoromethylpyrazoles, depending on the substitution of the diketone. Dehydration of the hydroxydihydropyrazoles can be effected with sulphuric acid in acetic acid to give the regioisomeric 3-substituted-1-(4-methylquinolin-2-yl)-5-trifluoromethylpyrazoles. In contrast, the reaction of two 4-hydrazinoquinolines with 1,1,1-trifluoropentane-2,4-dione afforded a different isolable intermediate, the corresponding hydrazone formed at the 4-carbonyl. Dehydration gave the 1-(substituted-quinolin-4-yl)-3-methyl-5-trifluoromethylpyrazoles. The regioisomeric identity of the pyrazoles was established using ^{19}F NMR. © Elsevier Science S.A.

Keywords: Pyrazole; Hydrazinoquinoline; Trifluoromethyl-β-diketone; Cyclisation; ^{19}F NMR

1. Introduction

Recent investigations from our laboratory have shown that the products obtained by treating 2- and 4-hydrazinoquinolines with β-dicarbonyl compounds are the pyrazoles [1,2], instead of the erroneously reported diazepines [3,4]. In continuation of this work, we focused our attention on the reaction of these hydrazines with trifluoromethyl 1,3-diketones. Such a study assumes greater significance in view of the current interest in the development and application of compounds bearing trifluoromethyl groups as pharmaceuticals and agrochemicals [5–7].

2. Results and discussion

In general, the reaction of a monosubstituted hydrazine with unsymmetrical β-diketones can result in the formation of isomeric pyrazoles, depending on the site of initial nucleophilic attack [8–10]. In the first part of the present study, the reaction of 2-hydrazino-4-methylquinoline **1** with the aliphatic trifluoromethyl-β-diketones **2a,b** was investigated. The sole products were identified as the 5-hydroxy-5-trifluoromethyl-4,5-dihydropyrazoles **3a,b**, respectively. However, similar treatment of the aryl trifluoromethyl-β-diketones **2c,d** with **1** in refluxing ethanol gave a mixture of the corresponding 5-hydroxy-5-trifluoromethyl-4,5-dihydropyrazoles **3c,d** and the 3-trifluoromethylpyrazoles **4c,d** (Scheme 1). No traces of **4a,b** were detected in the reaction mixtures. The hydroxydihydropyrazoles **3a–d** were only converted to the aromatic pyrazoles **5a–d** on treatment with sulphuric acid in acetic acid at elevated temperature. These observations, which raise questions of the initial site of nucleophilic attack on the diketone and rate of dehydration/aromatisation of the pyrazole, can be rationalised as follows.

As it is unlikely that the initial nucleophilic attack involves the secondary nitrogen of the hydrazinoquinoline, the first approach must be as shown in Scheme 2. It has been shown [11,12] that **2a–d** exist substantially in the enol form and that the direction of enolisation is largely towards $COCF_3$. Furthermore, there have been reports that these unsymmetrical diketones **2a–d** react with water [13], alcohols [13,14], ethanethiol [15] and pyrrolidine [15] to give the adducts at the $COCF_3$ carbonyl, probably through addition to the appropriate enol. It is therefore likely that, in the present study, the reaction proceeds via conjugate addition of the terminal nitrogen of the hydrazinoquinoline into the two enols, as shown in Scheme 2. Subsequent cyclisation affords the isomeric dihydroxytetrahydropyrazoles. One molecule of water is then eliminated to give the hydroxydihydropyrazoles; further

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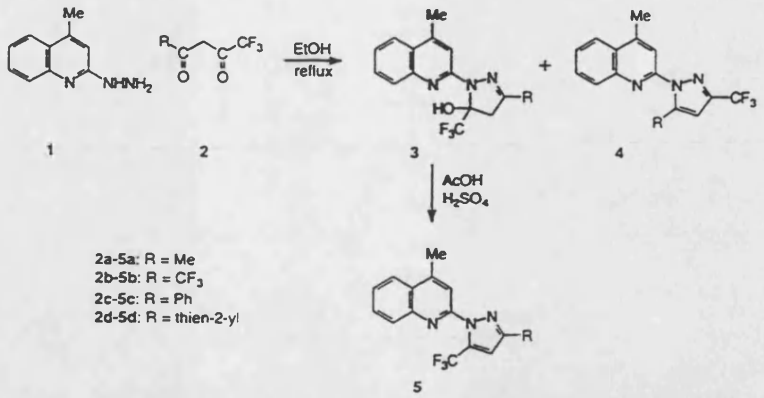
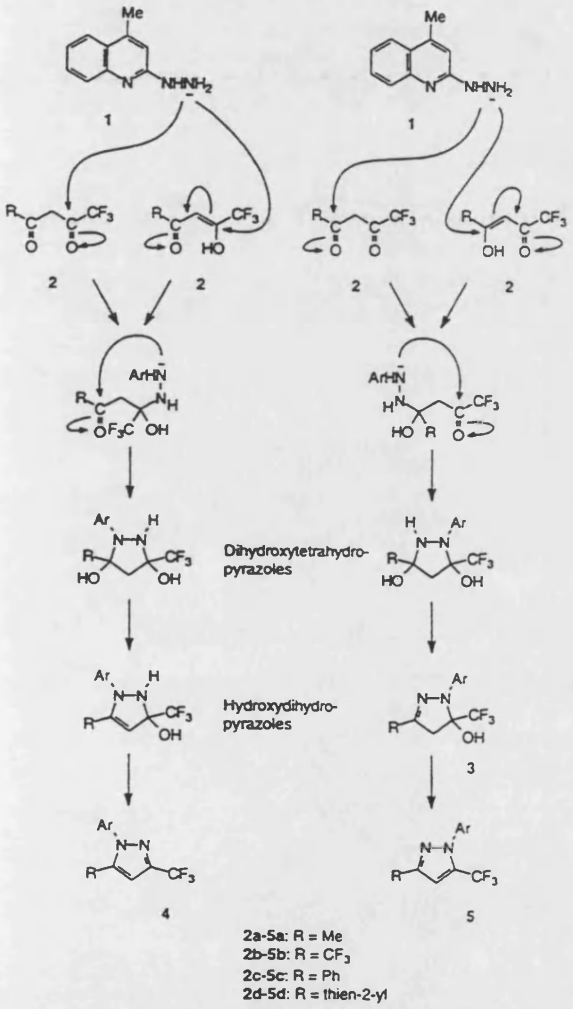


Scheme 1. Reaction of 2-hydrazino-4-methylquinoline 1 with trifluoromethyl β-diketones 2a–d.

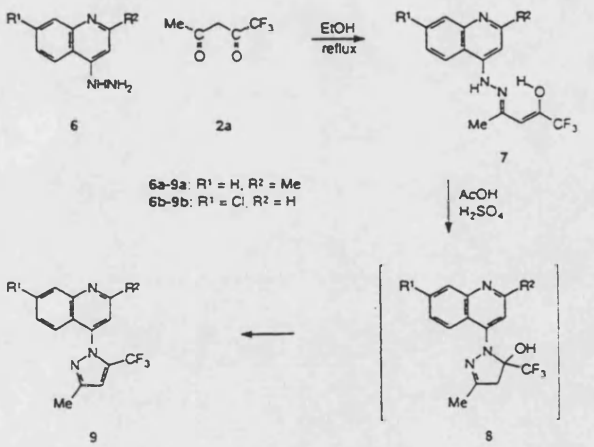


Scheme 2. Possible mechanistic routes for the reaction of 2-hydrazino-4-methylquinoline 1 with trifluoromethyl β-diketones 2a–d.

elimination of water yields the aromatic pyrazoles 4 and 5. The ratio of yields of these two regioisomers depends on the proportion of the two enols at equilibrium. For β-diketone **2a**, where R is aliphatic, the $COCF_3$ carbonyl is predominantly enolised, leading eventually to products **3a** and **5a**. In contrast, where R is aromatic (**2c,d**), this enol is cross-conjugated between the C=C and the arene, whereas the alternative enol is fully conjugated in the system Ar–C=C–C=O. Thus, there is now a significant amount of the minor enol present in the reaction mixture and attack can take place, leading to significant amounts of the regioisomer **4**. It should be noted that this effect runs counter to steric influences.

The hydroxydihydropyrazoles **3** can be isolated as stable crystalline solids from the initial reaction mixture but the regioisomeric hydroxydihydropyrazoles are converted directly in boiling ethanol to the pyrazoles **4**. These observations must reflect the relative ease of elimination of the second molecule of water in the overall condensation. Presumably, as the OH or OH_2 leaves, positive charge builds up at C-5 and the electron-withdrawing trifluoromethyl group destabilises this build-up. This effect has been noted [16] for the reaction of 1,1,1,5,5,5-hexafluoropentane-2,4-dione **2b** with other aryl and acyl hydrazines.

In contrast (Scheme 3), when the hydrazine was translocated to the 4-position of the quinoline and substrates **6a,b** were allowed to react with **2a** under the standard conditions (boiling ethanol), the stable crystalline hydrazones **7a,b** were



Scheme 3. Reaction of 4-hydrazinoquinolines **6a,b** with 1,1,1-trifluoropentane-2,4-dione 2a.

isolated. Elimination of the second molecule of water was effected only by treatment with sulphuric acid in hot acetic acid, reflecting again the relative difficulty of this elimination from a 5-hydroxy-5-trifluoromethyldihydropyrazole. These observations indicate that elimination of the first molecule of water precedes the cyclisation with these substrates **6**. As expected from the studies with **1** and **2a**, there was no evidence of formation of other regioisomers. We were unable to isolate the corresponding hydroxydihydropyrazoles **8a,b** from the reactions, although it is unclear why the point of attachment to the quinoline should have such an effect on the course of the reaction. Hydrazones related to **7** are rarely reported in the literature [17,18] and only those too without direct spectroscopic evidence for their structure.

The structures of the pyrazoles and the intermediates were deduced from their NMR spectra. For example, the ^{1}H NMR spectrum of **3a** displays the signal for the pyrazole 3-Me at δ 2.09 and the methylene protons resonate as an AB system at δ 3.17 and δ 3.34, with a geminal coupling constant ^2J 18.6 Hz. In the regioisomeric hydroxydihydropyrazole (leading to **4a**), the methyl group would resonate at δ ca. 1.5. The same pattern was evident for the analogues **3b–d**. We have previously shown this AB pattern to be characteristic in a series of 5-hydroxy-3,5-bis(trifluoromethyl)-4,5-dihydropyrazoles [16]. Structures **3a–d** were confirmed by mass spectroscopic studies in which molecular ions were observed corresponding to the hydroxydihydropyrazoles, rather than the dihydroxytetrahydropyrazoles or pyrazoles. Further support for the regioisomeric identity of **3a–d** was given by ^{13}C NMR (Table 1). The presence of the trifluoromethyl group makes assignment of the signals straightforward. For example, for **3a**, pyrazole C-3 (sp^2) resonates as a singlet at δ 151.37, C-4 as a singlet at δ 47.90 and C-5 as a quartet ($^2J_{C-F}$ 34.1 Hz) at δ 93.09. In **3b**, which contains two trifluoromethyl groups, pyrazole C-3 appears as a quartet ($^2J_{C-F}$ 39.7 Hz) at δ 140.31, a chemical shift which is appropriate for a sp^2 carbon. Thus the trifluoromethyl group must be attached to the sp^3 carbon in **3a** and the regioisomer must be as shown.

For the hydrazones **7a,b**, the ^{1}H NMR data lend support to the acyclic hydrazone–enol structure as follows. Firstly, no signal is seen at δ 3.5 but rather a singlet from the enol =CH– appears at δ 5.42 for **7a**, ruling out a hydrazone–ketone structure. Secondly, the NH and OH protons of **7a** resonate at δ 11.40 and δ 14.32, respectively, which indicate both that an OH is present and that there is strong intramolecular hydrogen-bonding. Similar observations were recorded for **7b**. The enol form of the hydrazones **7a,b** was further suggested by

Table 1
^{13}C NMR data for hydroxydihydropyrazoles **3a–d**

Carbon atom	3a	3b	3c	3d
Pyrazole carbons				
C-3	151.37	140.31 (q, $^2J_{C-F}$ = 39.7 Hz)	150.19	146.07
C-4	47.90	41.95	44.14	44.74
C-5	93.09 (q, $^2J_{C-F}$ = 34.1 Hz)	94.62 (q, $^2J_{C-F}$ = 34.2 Hz)	93.51 (q, $^2J_{C-F}$ = 33.1 Hz)	93.50 (q, $^2J_{C-F}$ = 33.0 Hz)
Quinoline carbons				
C-2	155.54	154.39	155.24	155.03
C-3	112.90	112.53	112.79	112.75
C-4	144.82	144.43	144.79	144.72
C-5	123.83	125.02	124.02	124.04
C-6	123.70	123.83	123.78	123.76
C-7	126.96	127.37	129.83	128.24
C-8	129.70	130.19	130.02	129.82
C-4a	124.45	125.21	124.62	124.62
C-8a	146.65	147.87	146.84	146.91
Other carbons				
quinoline-CH_3	18.87	18.90	19.04	18.99
pyrazole-CF_3	123.97	123.34	123.95	123.86
	(q, $^1J_{C-F}$ = 287.6 Hz)	(q, $^1J_{C-F}$ = 287.5 Hz)	(q, $^1J_{C-F}$ = 287.7 Hz)	(q, $^1J_{C-F}$ = 288.8 Hz)
pyrazole 3-R	15.53 (Me)	119.82 (q, $^1J_{C-F}$ = 269.9 Hz) (CF_3)	130.97 (Ph C-1)	134.43 (thiophene C-2)
			126.14 (Ph C-2,6)	128.00 (thiophene C-3)
			128.75 (Ph C-3,5)	126.98 (thiophene C-4)
			127.05 (Ph C-4)	127.60 (thiophene C-5)

the ^{13}C NMR data, in contrast to the isomeric hydroxydihydropyrazoles **8a,b**. Characteristic signals were seen for **7a** at δ 169.60 ($^2J_{C-F}$ 31.2 Hz, =C–CF_3), δ 118.54 ($^1J_{C-F}$ 286.6 Hz, CF_3) and δ 87.84 (=CH–). No CH_2 signals were seen in the 135 DEPT spectrum, ruling out hydrazone–ketone and hydroxydihydropyrazole structures. The pattern of chemical shifts and multiplicities for the C-5 unit are very similar to those reported [19] for the enol form of 1,1,1-trifluoropentane-2,4-dione **2a**.

The pyrazoles **4c,d**, **5a–d** and **9** were characterised by a signal in the range δ 6.63–δ 7.21 for the aromatic pyrazole 4-H. It is interesting to note that the presence of trifluoromethyl at pyrazole C-5 causes the 4-H to resonate further downfield as compared with trifluoromethyl at pyrazole C-3 ($\Delta\delta$ 0.42 for **4c/5c**, $\Delta\delta$ 0.21 for **4d/5d**).

Complete analysis of the ^{13}C spectra of **4c,d**, **5a–d** and **9** (Table 2) was achieved by comparison with reported chemical shifts for pyrazoles [20–22] and quinolines [23] and by DEPT experiments (Table 2). The CF_3 carbons resonated at ca. δ 120 with $^1J_{C-F}$ ca. 270 Hz. The C–CF_3 carbons resonated as quartets at δ ca. 143 and δ ca. 133 for pyrazole C-3 and pyrazole C-5, respectively. In the corresponding 1-(quinolin-2-yl)-3,5-dimethylpyrazoles [1], C-3 and C-5 resonate at δ ca. 150 and δ ca. 142, respectively. Thus it can be seen that replacement of CH_3 by CF_3 results in shielding of these ring carbons by ca. 8 ppm.

Finally, ^{19}F NMR has been found to be an elegant method for assigning the trifluoromethyl hydroxydihydropyrazole, hydrazone and pyrazole structures (Table 3). The hydroxydihydropyrazoles **3a–d** exhibited signals at δ ca. −81 for the 5-CF_3, in contrast to δ −67.39 for the 3-CF_3 of **3b**. In contrast, the trifluoromethyl group of the hydrazones **7a,b** resonate at δ ca. −75. Isomeric trifluoromethylpyrazoles can easily be distinguished by their ^{19}F spectra. The 5-CF_3 resonates at δ ca. −58, in contrast to the more upfield resonance of the 3-CF_3 at δ ca. −62.

3. Experimental details

Melting points were determined using open capillaries in a sulphuric acid bath and are uncorrected. ^1H NMR spectra were obtained at 270 MHz and 400 MHz, ^{13}C spectra at 67.5 MHz and 100 MHz and ^{19}F spectra at 376 MHz, using Jeol GX270 and Jeol EX400 instruments, using deuteriochloroform as solvent, unless otherwise noted. The internal standard for the ^{19}F spectra was fluorotrichloromethane, setting the $CF^{35}Cl_3$ signal as δ 0.00. ^{13}C and ^{19}F NMR data for **3a–d**, **4c,d**, **5a–d** and **9a,b** are presented in Tables 1–3, respectively. The stationary phase for chromatography was silica gel. High resolution mass spectra were measured in the EI mode on a Kratos MS-50 spectrometer. Elemental analyses were performed at RSIC, Chandigarh, India. 2-Hydrazino-4-methylquinoline **1** [24], 4-hydrazino-2-methylquinoline **6a** [25] and 7-chloro-4-hydrazinoquinoline **6b** [26] were prepared by procedures described in the literature.

3.1. 5-Hydroxy-3-methyl-1-(4-methylquinolin-2-yl)-5-trifluoromethyl-4,5-dihydropyrazole (3a)

1,1,1-Trifluoropentane-2,4-dione **2a** (770 mg, 5 mmol) was boiled under reflux with 2-hydrazino-4-methylquinoline **1** (865 mg, 5 mmol) in ethanol (50 ml) for 3 h. Evaporation of the solvent and recrystallisation (ethanol) provided the hydroxydihydropyrazole **3a** (1.20 g, 78%) as a pale yellow solid, m.p. 113–114 °C. ^1H NMR δ: 2.09 (s, 3 H, pyrazole-Me); 2.62 (s, 3 H, quinoline-Me); 3.17 (d, 1 H, J = 18.6 Hz, pyrazole 4-H); 3.34 (d, 1 H, J = 18.6 Hz, pyrazole 4-H); 7.35 (ddd, 1 H, quinoline 6-H); 7.41 (s, 1 H, quinoline 3-H); 7.57 (ddd, 1 H, quinoline 7-H); 7.70 (d, 1 H, quinoline 5-H); 7.82 (d, 1 H, quinoline 8-H) ppm. MS m/z: 309.1092 (M) ($C_{15}H_{14}F_3N_3O$ requires: 309.1089); 291.0991 (M − H_2O) ($C_{15}H_{12}F_3N_3$ requires: 291.0983) (100%). Analysis: Found: N, 13.54%. $C_{15}H_{14}F_3N_3O$ requires: N, 13.59%.

3.2. 3,5-Bis(trifluoromethyl)-5-hydroxy-1-(4-methylquinolin-2-yl)-4,5-dihydropyrazole (3b)

1,1,1,5,5,5-Hexafluoropentane-2,4-dione **2b** (1.04 mg, 5 mmol) was treated with **1** (865 mg, 5 mmol), as for the synthesis of **3a**, to give the hydroxydihydropyrazole **3b** (1.35 g, 75%) as a pale yellow solid, m.p. 135–136 °C. ^1H NMR δ: 2.65 (s, 3 H, quinoline-Me); 3.42 (d, 1 H, J = 19.0 Hz, pyrazole 4-H); 3.60 (d, 1 H, J = 19.0 Hz, pyrazole 4-H); 7.42 (s, 1 H, quinoline 3-H); 7.43 (ddd, 1 H, quinoline 6-H); 7.62 (ddd, 1 H, quinoline 7-H); 7.74 (d, 1 H, quinoline 5-H); 7.87 (d, 1 H, quinoline 8-H) ppm. MS m/z: 363.0791 (M) ($C_{15}H_{11}F_6N_3O$ requires: 363.0806); 345.0703 (M − H_2O) ($C_{15}H_9F_6N_3$ requires: 345.0701); 294.0858 (M − CF_3) ($C_{14}H_{11}F_3N_3O$ requires: 294.0854) (100%). Analysis: Found: N, 11.49%. $C_{15}H_{11}F_6N_3O$ requires: N, 11.57%.

3.3. 5-Hydroxy-1-(4-methylquinolin-2-yl)-3-phenyl-5-trifluoromethyl-4,5-dihydropyrazole (3c) and 1-(4-methylquinolin-2-yl)-5-phenyl-3-trifluoromethylpyrazole (4c)

1-Phenyl-4,4,4-trifluorobutane-1,3-dione **2c** (1.08 g, 5 mmol) was boiled under reflux with 2-hydrazino-4-methylquinoline **1** (865 mg, 5 mmol) in ethanol (50 ml) for 3 h and the solvent was evaporated. Chromatography (light petroleum (b.p. 60–80 °C):benzene (CAUTION) 4:1) afforded the hydroxydihydropyrazole **3c** (1.15 g, 62%) as an off-white solid, m.p. 137–138 °C. ^1H NMR δ: 2.68 (s, 3 H, quinoline-Me); 3.63 (d, 1 H, J = 18.3 Hz, pyrazole 4-H); 3.77 (d, 1 H, J = 18.3 Hz, pyrazole 4-H); 7.24–7.44 (m, 4 H, quinoline 6-H + Ph 3,4,5-H_3); 7.58–7.62 (m, 2 H, quinoline 3,7-H_2); 7.73–7.77 (m, 3 H, quinoline 5-H + Ph 2,6-H_2); 7.86 (d, 1 H, quinoline 8-H) ppm. Analysis: Found: N, 11.42%. $C_{20}H_{16}F_3N_3O$ requires: N, 11.32%. Further elution (light petroleum (b.p. 60–80 °C):benzene 1:1) afforded the

Table 2
^{13}C NMR data for compounds 4c,d, 5a–d and 9a,b

Carbon atom	4c	4d	5a	5b	5c	5d	9a	9b
Pyrazole carbons								
C-3	143.71 (q, $^2J_{C-F}$ = 36.8 Hz)	143.58 (q, $^2J_{C-F}$ = 38.6 Hz)	149.47	142.05 (q, $^2J_{C-F}$ = 38.6 Hz)	149.49	147.61	149.99	150.39
C-4	106.72	106.83	111.57	108.36	109.04	108.97	108.35	108.80
C-5	145.82	139.53	133.12 (q, $^2J_{C-F}$ = 40.7 Hz)	133.26 (q, $^2J_{C-F}$ = 42.9 Hz)	133.82 (q, $^2J_{C-F}$ = 41.0 Hz)	133.84 (q, $^2J_{C-F}$ = 40.5 Hz)	134.20 (q, $^2J_{C-F}$ = 38.6 Hz)	134.20 (q, $^2J_{C-F}$ = 40.0 Hz)
Quinoline carbons								
C-2	150.11	149.88	149.65	147.69	151.78	149.27	158.97	151.16
C-3	116.96	117.49	118.17	113.47	114.65	114.62	119.90	119.01
C-4	145.98	145.85	146.10	144.80	146.00	145.98	143.11	142.98
C-5	126.94	126.94	126.36	126.25	125.94	125.43	127.09	124.44
C-6	123.67	123.82	123.64	122.76	123.69	123.71	122.57	128.59
C-7	128.99	129.28	129.56	128.75	128.86	129.56	128.81	136.49
C-8	129.67	130.22	129.91	129.36	129.58	130.02	130.35	129.10
C-4a	127.46	127.42	127.22	126.75	127.35	127.36	123.45	123.52
C-8a	147.92	148.19	147.41	147.40	147.52	147.23	149.11	149.86
Other carbons								
quinoline-CH_3	18.88 (4-CH_3)	18.86 (4-CH_3)	18.86 (4-CH_3)	17.92 (4-CH_3)	18.99 (4-CH_3)	18.99 (4-CH_3)	25.30 (2-CH_3)	
pyrazole 3-CH_3			13.47				13.38	13.38
pyrazole 3-CF_3	121.17 (q, $^1J_{C-F}$ = 270.3 Hz)	121.02 (q, $^1J_{C-F}$ = 268.4 Hz)		118.30 (q, $^1J_{C-F}$ = 268.8 Hz)				
pyrazole 5-CF_3			120.14 (q, $^1J_{C-F}$ = 267.7 Hz)	119.36 (q, $^1J_{C-F}$ = 268.9 Hz)	120.07 (q, $^1J_{C-F}$ = 268.0 Hz)	119.92 (q, $^1J_{C-F}$ = 268.4 Hz)	119.46 (q, $^1J_{C-F}$ = 268.4 Hz)	119.50 (q, $^1J_{C-F}$ = 270.0 Hz)
Ph or thiophene	130.13 (Ph C-1) 128.11 (Ph C-2,6) 130.00 (Ph C-3,5) 128.68 (Ph C-4)	130.00 (thiophene C-2) 129.63 (thiophene C-3) 127.22 (thiophene C-4) 127.79 (thiophene C-5)			131.55 (Ph C-1) 126.49 (Ph C-2,6) 130.00 (Ph C-3,5) 128.81 (Ph C-4)	134.50 (thiophene C-2) 127.68 (thiophene C-3) 126.03 (thiophene C-4) 126.54 (thiophene C-5)		

Table 3
^{19}F NMR data for compounds 3a–d, 4c,d, 5a–d and 9a,b

	3a	3b	3c	3d	4c	4d	5a	5b	5c	5d	9a	9b
3-CF_3		67.39			62.80	62.87		63.16				
5-CF_3	81.72	81.64	81.63	81.58			58.15	58.49	58.13	58.25	58.94	58.80

pyrazole **4c** (185 mg, 10%) as an off-white solid, m.p. 110–111 °C. ^{1}H NMR δ 2.72 (d, 3 H, J = 0.9 Hz, quinoline-Me); 6.79 (s, 1 H, pyrazole 4-H); 7.24–7.36 (m, 5 H, Ph-H_5); 7.52–7.69 (m, 4 H, quinoline 3,5,6,7-H_4); 7.98 (d, 1 H, quinoline 8-H) ppm. Analysis: Found: N, 11.86%. $C_{20}H_{14}F_3N_3$ requires: N, 11.90%.

3.4. 5-Hydroxy-1-(4-methylquinolin-2-yl)-3-(thien-2-yl)-5-trifluoromethyl-4,5-dihydropyrazole (3d) and 1-(4-methylquinolin-2-yl)-5-(thien-2-yl)-3-trifluoromethylpyrazole (4d)

1-(Thien-2-yl)-4,4,4-trifluorobutane-1,3-dione **2d** (1.11 g, 5 mmol) was boiled under reflux with 2-hydrazino-4-methylquinoline **1** (865 mg, 5 mmol) in ethanol (50 ml) for 3 h and the solvent was evaporated. Chromatography (light petroleum (b.p. 60–80 °C):benzene (CAUTION) 4:1) afforded the hydroxydihydropyrazole **3d** (1.15 g, 61%) as a pale solid, m.p. 123–124 °C. ^{1}H NMR δ: 2.66 (d, 3 H, J = 0.7 Hz, quinoline-Me); 3.61 (d, 1 H, J = 18.1 Hz, pyrazole 4-H); 3.77 (d, 1 H, J = 18.1 Hz, pyrazole 4-H); 7.06 (dd, 1 H, thiophene 4-H); 7.20 (dd, 1 H, thiophene 3-H); 7.34–7.41 (m, 2 H, quinoline 6-H + thiophene 5-H); 7.52 (s, 1 H, quinoline 3-H); 7.59 (ddd, 1 H, quinoline 7-H); 7.72 (d, 1 H, quinoline 5-H); 7.84 (d, 1 H, quinoline 8-H); 9.43 (br, 1 H, OH) ppm. Analysis: Found: N, 11.27%. $C_{18}H_{14}F_3N_3OS$ requires: N, 11.14%. Further elution (light petroleum (b.p. 60–80 °C):benzene 1:1) afforded the pyrazole **4d** (150 mg, 8%) as a pale solid, m.p. 75–76 °C. ^{1}H NMR δ: 2.73 (d, 3 H, J = 0.7 Hz, quinoline-Me); 6.87 (s, 1 H, pyrazole 4-H); 6.97 (dd, 1 H, thiophene 4-H); 7.22 (dd, 1 H, thiophene 3-H); 7.33 (dd, 1 H, thiophene 5-H); 7.57–7.63 (m, 2 H, quinoline 3,6-H_2); 7.72 (ddd, 1 H, quinoline 7-H); 7.96–8.03 (m, 2 H, quinoline 5,8-H_2) ppm. Analysis: Found: N, 11.73%. $C_{18}H_{12}F_3N_3S$ requires: N, 11.70%.

3.5. 3-Methyl-1-(4-methylquinolin-2-yl)-5-trifluoromethylpyrazole (5a)

The hydroxydihydropyrazole **3a** (927 mg, 3 mmol) was boiled under reflux with concentrated sulphuric acid (0.2 ml) in acetic acid (20 ml) for 5 h. The mixture was poured into ice-water and was extracted thrice with dichloromethane. The combined organic extracts were washed with aqueous sodium hydrogen carbonate solution and with water and were dried (sodium sulphate). The solvent was evaporated and the residue was recrystallised from ethanol to give the pyrazole **5a** (788 mg, 85%) as a pale yellow solid, m.p. 103–104 °C. ^{1}H NMR δ: 2.39 (s, 3 H, pyrazole-Me); 2.74 (s, 3 H, quinoline-Me); 6.70 (s, 1 H, pyrazole 4-H); 7.54 (ddd, 1 H, quinoline 6-H); 7.71 (ddd, 1 H, quinoline 7-H); 7.83 (s, 1 H, quinoline 3-H); 7.97 (d, 1 H, quinoline 5-H); 8.03 (d, 1 H, quinoline 8-H) ppm. MS m/z: 291.0987 (M) ($C_{15}H_{12}F_3N_3$ requires: 291.0983) (100%); 222.1025 (M − CF_3) ($C_{14}H_{12}N_3$ requires: 222.1031). Analysis: Found: N, 14.41%. $C_{15}H_{12}F_3N_3$ requires: N, 14.43%.

3.6. 3,5-Bis(trifluoromethyl)-1-(4-methylquinolin-2-yl)-pyrazole (5b)

The hydroxydihydropyrazole **3b** was treated with sulphuric acid and acetic acid, as for the synthesis of **5a**, to give the *pyrazole* **5b** (80%) as a pale yellow solid, m.p. 93–94 °C. ^{1}H NMR δ: 2.76 (s, 3 H, quinoline-Me); 7.15 (s, 1 H, pyrazole 4-H); 7.60 (t, 1 H, quinoline 6-H); 7.74 (t, 1 H, quinoline 7-H); 7.85 (s, 1 H, quinoline 3-H); 8.00 (d, 1 H, quinoline 5-H); 8.04 (d, 1 H, quinoline 8-H) ppm. MS m/z: 345.0692 (M) ($C_{15}H_9F_6N_3$ requires: 345.0700) (100%). Analysis: Found: N, 12.13%. $C_{15}H_9F_6N_3$ requires: N, 12.17%.

3.7. 1-(4-Methylquinolin-2-yl)-3-phenyl-5-trifluoromethylpyrazole (5c)

The hydroxydihydropyrazole **3c** was treated with sulphuric acid and acetic acid, as for the synthesis of **5a**, to give the pyrazole **5c** (75%) as a pale yellow solid, m.p. 109–110 °C. ^{1}H NMR δ: 2.76 (d, 3 H, J = 0.9 Hz, quinoline-Me); 7.21 (s, 1 H, pyrazole 4-H); 7.36–7.49 (m, 3 H, Ph 3,4,5-H_3); 7.55 (ddd, 1 H, quinoline 6-H); 7.72 (ddd, 1 H, quinoline 7-H); 7.90–7.98 (m, 4 H, quinoline 3,5-H_2 + Ph 2,6-H_2); 8.05 (d, 1 H, quinoline 8-H) ppm. Analysis: Found: N, 12.02%. $C_{20}H_{14}F_3N_3$ requires: N, 11.90%.

3.8. 1-(4-Methylquinolin-2-yl)-3-(thien-2-yl)-5-trifluoromethylpyrazole (5d)

The hydroxydihydropyrazole **3d** was treated with sulphuric acid and acetic acid, as for the synthesis of **5a**, to give the pyrazole **5d** (70%) as a pale yellow solid, m.p. 116–117 °C. ^{1}H NMR δ: 2.76 (d, 3 H, J = 0.7 Hz, quinoline-Me); 7.08–7.11 (m, 2 H, pyrazole 4-H + thiophene 4-H); 7.34 (dd, 1 H, thiophene 5-H); 7.45 (dd, 1 H, thiophene 3-H); 7.55 (ddd, 1 H, quinoline 6-H); 7.72 (t, 1 H, quinoline 7-H); 7.94 (s, 1 H, quinoline 3-H); 7.97 (d, 1 H, quinoline 5-H); 8.04 (d, 1 H, quinoline 8-H) ppm. Analysis: Found: N, 11.82%. $C_{18}H_{12}F_3N_3S$ requires: N, 11.70%.

3.9. 4-(N'-(3-Hydroxy-1-methyl-4,4,4-trifluorobut-2-enylidine))hydrazino-2-methylquinoline (7a)

1,1,1-Trifluoropentane-2,4-dione **2a** (770 mg, 5 mmol) was boiled under reflux with **6a** (865 mg, 5 mmol) in ethanol (50 ml) for 6 h. Evaporation of the solvent and recrystallisation (methanol) afforded the hydrazone **7a** (1.10 g, 70%) as a yellow solid, m.p. 239–240 °C. ^{1}H NMR ($(CD_3)_2SO$) δ: 2.32 (s, 3 H, =CMe); 2.36 (s, 3 H, quinoline-Me); 5.42 (s, 1 H, =CH); 5.90 (s, 1 H, quinoline 3-H); 7.24 (t, 1 H, quinoline 7-H); 7.34 (d, 1 H, quinoline 5-H); 7.53 (ddd, 1 H, quinoline 6-H); 8.15 (dd, 1 H, quinoline 8-H); 11.40 (s, 1 H, NH); 14.32 (s, 1 H, OH) ppm. ^{13}C NMR δ: 87.84 (–CH=); 118.54 (q, $^1J_{C-F}$ = 286.6 Hz, CF3); 169.60 (q, $^2J_{C-F}$ = 31.2 Hz, CCF3) ppm. ^{19}F NMR δ: −74.79 (s) ppm. Analysis: Found: N, 13.52%. $C_{15}H_{14}F_3N_3O$ requires: N, 13.59%.

3.10. 7-Chloro-4-(N'-(3-Hydroxy-1-methyl-4,4,4-trifluorobut-2-enylidine))hydrazinoquinoline (7b)

1,1,1-Trifluoropentane-2,4-dione **2a** (770 mg, 5 mmol) was treated with **6b** (965 mg, 5 mmol), as for the synthesis of **7a**, to afford the hydrazone **7b** (1.18 g, 72%) as a yellow solid, m.p. 255–256 °C. ^{1}H NMR δ: 2.38 (s, 3 H, =CMe); 5.51 (s, 1 H, =CH); 6.11 (d, 1 H, quinoline 3-H); 7.31 (dd, 1 H, quinoline 6-H); 7.40 (d, 1 H, quinoline 5-H); 7.74 (d, 1 H, quinoline 2-H); 8.20 (dd, 1 H, quinoline 8-H); 11.49 (s, 1 H, NH); 14.30 (s, 1 H, OH) ppm. ^{13}C NMR δ ((CD3)2SO): 87.79 (–CH=); 118.35 (q, $^1J_{C-F}$ 286.4 Hz, CF3); 170.23 (q, $^2J_{C-F}$ 30.8 Hz, CCF3) ppm. ^{19}F NMR δ: −74.37 (s) ppm. MS m/z: 331/329 (M). Analysis: Found: N, 12.71%. $C_{14}H_{11}ClF_3N_3O$: N, 12.73%.

3.11. 3-Methyl-1-(2-methylquinolin-4-yl)-5-trifluoromethylpyrazole (9a)

The hydrazone **7a** (927 mg, 3 mmol) was boiled under reflux in acetic acid (30 ml) for 4 h. Evaporation of the solvent and recrystallisation (ethanol) afforded the pyrazole **9a** (650 mg, 75%) as a pale yellow solid, m.p. 135–136 °C. ^{1}H NMR δ: 2.37 (s, 3 H, pyrazole-Me); 2.72 (s, 3 H, quinoline-Me); 6.63 (s, 1 H, pyrazole 4-H); 7.25 (s, 1 H, quinoline 3-H); 7.29 (d, 1 H, quinoline 5-H); 7.41 (t, 1 H, quinoline 6-H); 7.65 (t, 1 H, quinoline 7-H); 8.03 (d, 1 H, quinoline 8-H) ppm. Analysis: Found: N, 14.38%. $C_{15}H_{12}F_3N_3$ requires: N, 14.43%.

3.12. 1-(7-Chloroquinolin-4-yl)-3-methyl-5-trifluoromethylpyrazole (9b)

The hydrazone **7b** (988 mg, 3 mmol) was boiled under reflux in acetic acid (30 ml) for 4 h. Evaporation of the solvent and recrystallisation from ethanol afforded the pyrazole **9b** (670 mg, 72%) as a pale yellow solid, m.p. 78–79 °C. ^{1}H NMR δ: 2.43 (s, 3 H, pyrazole-Me); 6.74 (s, 1 H, pyrazole 4-H); 7.44 (d, 1 H, quinoline 3-H); 7.46 (d, 1 H, quinoline 5-H); 7.53 (dd, 1 H, quinoline 6-H); 8.23 (d, 1 H, quinoline 8-H); 9.04 (d, 1 H, quinoline 2-H) ppm. MS m/z 311/313 (M). Analysis: Found: N, 13.42%. $C_{14}H_9ClF_3N_3$ requires: N, 13.46%.

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Synthesis of 8-(ω-Aminoalkyl)theophyllines and their Use in Preparing Fluorescently Labelled Derivatives

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8-(ω-Aminoalkyl)theophyllines and Their Use in Preparing Fluorescently Labeled Derivatives for Applications in Immunoassay

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Reaction of alkane-1,ω-diamines with 6-chloro-1,3-dimethylpyrimidine-2,4-dione under carefully controlled conditions gives 6-(ω-aminoalkylamino)-1,3-dimethylpyrimidine-2,4-diones, which can be readily separated from traces of products of disubstitution after benzyloxycarbonyl protection. A sequence of nitrosation at the pyrimidine 5-position, thermal cyclization, and deprotection affords 8-(ω-aminoalkyl) derivatives of theophylline, an important drug in the treatment of asthma and related diseases. These 8-(ω-aminoalkyl)theophyllines can be coupled to fluorescein-5-isothiocyanate and to dansyl chloride, giving fluorescent derivatives of theophylline with applications in automated immunoassay of the drug in biofluids using the fluorescence capillary fill device.

INTRODUCTION

Theophylline (**1**; Scheme 1) is a drug that is commonly used as a bronchospasmolytic agent in the prevention and treatment of asthma, apnoea, and obstructive lung diseases and is an inhibitor of cyclic nucleotide phosphodiesterases (*1–3*). Drug-induced toxicity occurs at plasma concentrations not far above those required for therapy, and plasma concentrations vary greatly among individuals given the same dose. Rapid and accurate measurement of its concentration in biofluids is therefore necessary (*2, 3*). Current methods for the determination of theophylline in blood include ELISA (*4*), radioimmunoassay (*5*), and HPLC (*6*), although not all of these completely fulfill criteria of speed, ease, and low cost of assay. The fluorescence capillary fill device (FCFD) (*7–10*) overcomes these drawbacks. To develop an immunoassay for theophylline in whole blood using this device, we required fluorescent derivatives of theophylline that have good immunoreactivity with appropriate anti-theophylline antibodies. To be able to optimize both the photoproperties of the fluorophore and the length and lipophilicity of the link between theophylline and the fluorophore, a series of 8-(ω-aminoalkyl)theophyllines and related ethers was sought. Thus, a variety of widely used fluorescent groups bearing isothiocyanate and sulfonyl chloride electrophiles could be attached.

EXPERIMENTAL PROCEDURES

Chemical Synthesis. IR spectra were recorded on a Perkin-Elmer 782 spectrophotometer as potassium bromide disks, unless otherwise stated. NMR spectra were recorded using JEOL JNM GX270 and JNM EX400 spectrometers of samples in $(CD_3)_2SO$, except where stated. Chemical shifts were measured relative to tetramethylsilane as internal standard. Mass spectra were obtained using a VG7070E analytical mass spectrometer using electron impact (EI) or fast atom bombardment (FAB) techniques in the positive ion mode. Melting points are uncorrected. Dioxane refers to 1,4-dioxane. Chromatographic separations were carried out using Sorbsil C60 (0.040–0.063) silica gel. Solvents were evaporated under reduced pressure. Solutions in organic solvents were dried over magnesium sulfate. Experiments were conducted at ambient temperature unless otherwise stated. Chemicals were purchased from Aldrich Chemical Co. (Gillingham, U.K.), Sigma Chemical Co. (Gillingham, U.K.), and Maybridge Chemicals (Tintagel, U.K.).

Phenylmethyl N-[[[6-(1,1-Dimethylethoxy)carbonyl]amino]hexyl]carbamate (3). Phenylmethyl chloroformate (610 mg, 3.6 mmol) in CH_2Cl_2 (6 mL) was added during 30 min to 1,1-dimethylethyl *N*-(6-aminohexyl)carbamate hydrochloride (**2**) (900 mg, 3.6 mmol) and Et_3N (720 mg, 7.1 mmol) in CH_2Cl_2 (12 mL). The mixture was stirred for 24 h and was filtered. The filtrate was washed twice with water and once with hydrochloric acid (2 M) and dried. The solvent was evaporated to afford **3** as a white crystalline solid (960 mg, 77%): mp 190 °C; IR 3340, 1680, 1550 cm^{-1}; NMR δ ($CDCl_3$) 1.44 (17 H, m, $CH_2CH_2CH_2CH_2CH_2CH_2$ + Bu^t), 1.85 (1 H, br, NH), 3.11 (2 H, q, J = 6 Hz, CH_2N), 3.17 (2 H, m, CH_2N), 4.85 (1 H, br, NH), 5.09 (2 H, s, $PhCH_2$), 7.35 (5 H, s, Ar-H_5); MS (EI) 350 (M). Anal. Calcd for $C_{19}H_{30}N_2O_4$: C, 65.12; H, 8.63; N, 7.99. Found: C, 65.41; H, 8.61; N, 8.17.

Phenylmethyl N-(6-Aminohexyl)carbamate Hydrochloride (4). HCl was passed through **3** (960 mg, 2.7 mmol) in CH_2Cl_2 (100 mL) for 1 h. The solvent and excess reagent were evaporated to afford **4** as white crystals (680 mg, 99%): mp 177–178 °C [lit. (*11*) mp 177–178 °C]; IR 3470, 3340–3280, 1700, 1630 cm^{-1}; NMR δ ($CDCl_3$) 1.18 (4 H, m, 2 × CH_2), 1.35 (4 H, m, 2 × CH_2), 2.93 (2 H, t, J = 6 Hz, $CH_2N^+H_3$), 3.08 (2 H, q, J = 6 Hz, CH_2NH), 3.61 (3 H, br, N^+H_3), 5.07 (2 H, s, $PhCH_2$), 7.39 (5 H, s, Ar-H_5); MS (EI) 287 (M).

1,3-Dimethyl-6-[[[[6-(phenylmethoxy)carbonyl]amino]hexyl]amino]pyrimidine-2,4-dione (6a). 1,3-Dimethyl-6-chloropyrimidine-2,4-dione (**5**) (490 mg, 2.8 mmol), **4** (840 mg, 3.7 mmol), and Na_2CO_3 (600 mg, 5.7 mmol) were boiled under reflux in dioxane (8 mL) for 4 h. The cooled mixture was diluted with $CHCl_3$, and the solution was washed twice with water and once with saturated brine and was dried. The evaporation residue was recrystallized (EtOH) to give **6a** as yellow crystals (600 mg, 54%): mp 128–130 °C; IR 3360, 3280, 1700, 1630 cm^{-1}; NMR δ 0.90 (4 H, m, 2 × CH_2), 1.23 (4 H, m, 2 × CH_2), 3.15 (2 H, t, J = 6 Hz, CH_2N), 3.17 (2 H, t, J = 6 Hz, CH_2N), 3.06 (3 H, s, NMe), 3.23 (3 H, s, NMe), 4.21 (2 H,

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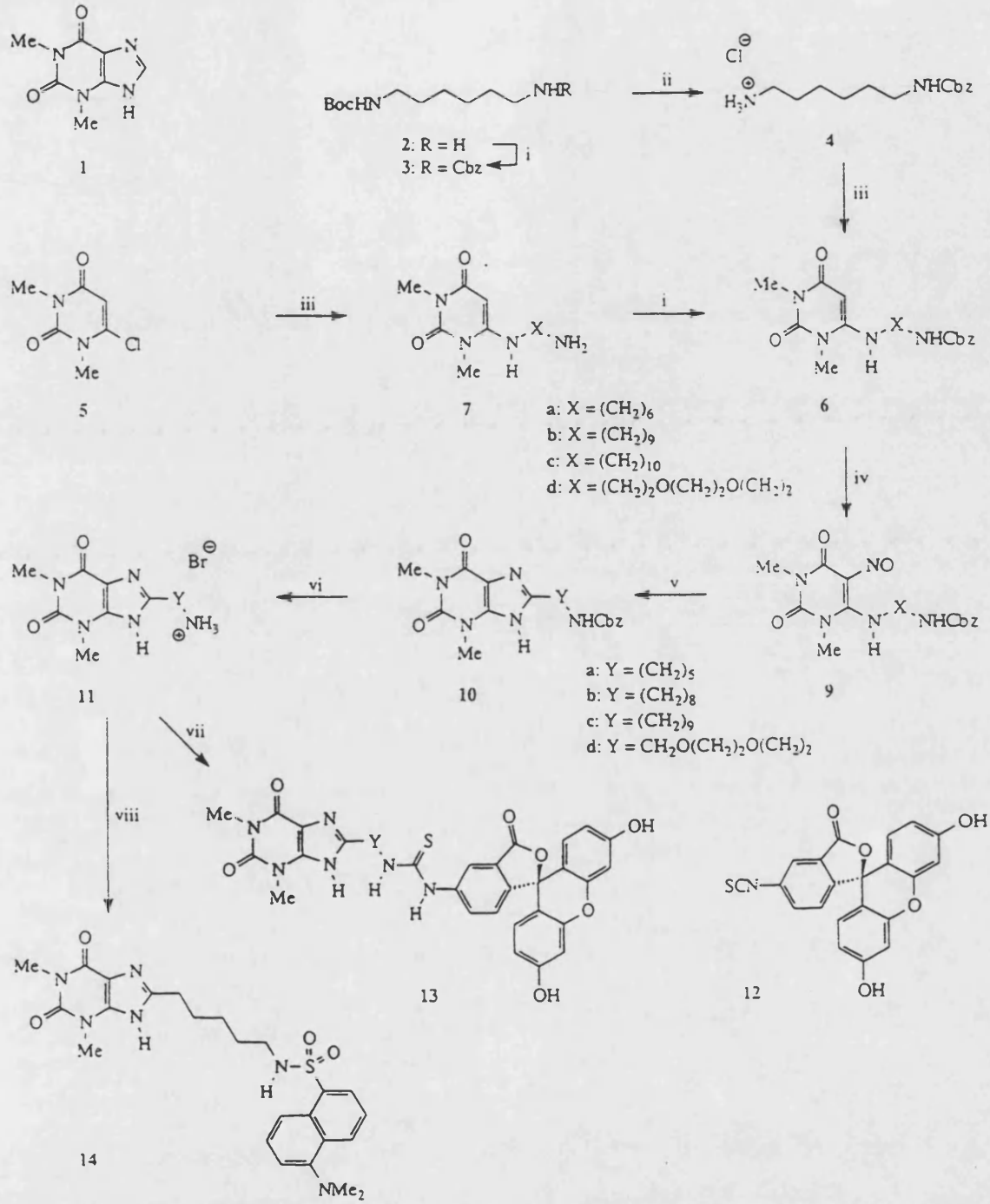
Scheme 1. Structures of Theophylline 1 and Fluorescein-5-isothiocyanate 12 and Synthetic Routes to the Fluorescent Theophylline Derivatives 13a–d and 14a



[a] Reagents: i, CbzCl, Et_3N, CH_2Cl_2; ii, HCl, CH_2Cl_2; iii, 5, 1,4-dioxane, Na_2CO_3, Δ; iv, $C_5H_{11}ONO$, HCl, EtOH; v, BuOH, Δ; vi, HBr, HOAc; vii, 12, K_2CO_3, dioxane, water, pH 9.0 ± 0.2; viii, 5-(dimethylamino)naphthalene-1-sulfonyl chloride, Na_2CO_3, dioxane, water, pH 9.5–10.0.

m, pyrimidine 5-H + NH), 4.69 (2 H, s, $PhCH_2$), 6.79 (5 H, m, Ph-H_5), 7.70 (1 H, br, NH); *m/z* (EI) 388 (M), 91 (100%). Anal. Calcd for $C_{20}H_{28}N_4O_4$: C, 61.82; H, 7.27; N, 14.43. Found: C, 61.85; H, 7.36; N, 14.3.

1,3-Dimethyl-6-[[[[9-(phenylmethoxy)carbonyl]-amino]nonyl]amino]pyrimidine-2,4-dione (6b). The chloropyrimidine **5** (1.00 g, 5.7 mmol) was boiled under reflux with nonane-1,9-diamine (1.81 g, 11 mmol) and Na_2CO_3 (1.21 g, 1.4 mmol) in EtOH (40 mL) for 3 h. The mixture was filtered and the solvent was evaporated. Recrystallization (EtOH) afforded 1,3-dimethyl-6-[(9-aminononyl)-amino]pyrimidine-2,4-dione (**7b**) as a white solid (1.09 g, 65%): mp 210-212 °C; IR 3500, 1660 cm^{-1}; NMR δ 1.29 [14 H, m, $(CH_2)_7$], 2.54 (2 H, m, CH_2NH_2), 3.03 (2 H, m, $NHCH_2$), 3.03 (3 H, s, NMe), 3.26 (3 H, s, NMe), 4.65 (1 H, br, NH), 6.72 (1 H , s, pyrimidine 5-H); *m/z* (EI) 297 (M), 159 (100%). This material (3.16 g, 11 mmol) was stirred with phenylmethyl chloroformate (1.91 g, 11 mmol) and Et_3N (2.16 g, 21 mmol) in CH_2Cl_2 (150 mL) for 24 h. The suspension was filtered, and the filtrate

was washed twice with water and once with hydrochloric acid (2 M) and was dried. Evaporation and chromatography ($CHCl_3$/EtOAc 4:1 — EtOAc) gave **6b** as white crystals (2.02 g, 43%): mp 105–106 °C; IR 3420, 1700, 1680 cm^{-1}; NMR δ ($CDCl_3$) 1.30 (4 H, br, 2 × CH_2), 1.50 (2 H, quintet, J = 7.3 Hz, CH_2), 1.66 (8 H, m, 4 × CH_2), 3.09 (2 H, q, J = 7.3 Hz, NCH_2), 3.18 (2 H, q, J = 6.3 Hz, NCH_2), 3.31 (3 H, s, NMe), 3.39 (3 H, s, NMe), 4.35 (1 H, br, NH), 4.79 (1 H, br, NH), 4.86 (1 H, s, 5-H), 5.09 (2 H, s, $PhCH_2$), 7.35 (5 H, s, Ph-H_5); MS (EI) 431 (M), 336 (M − $PhCH_2OH$), 91 (100%). Anal. Calcd for $C_{20}H_{28}N_4O_4$: C, 61.84; H, 7.27; N, 14.42. Found: C, 61.9; H, 7.35; N, 14.39.

1,3-Dimethyl-6-[[[[10-(phenylmethoxy)carbonyl]amino]decyl]amino]pyrimidine-2,4-dione (6c). The chloropyrimidine **5** (1.35 g, 7.7 mmol) was treated with decane-1,10-diamine (1.60 g, 9.3 mmol) as for the synthesis of **7b**, except that recrystallization was omitted, to give crude 1,3-dimethyl-6-[(10-aminodecyl)amino]pyrimidine-2,4-dione (**7c**) (1.24 g, 52%) as a white solid: mp 72-74 °C; IR 3400, 1660 cm^{-1}; NMR δ (CF_3CO_2D) 1.41 [16 H, m, $(CH_2)_8$], 1.82 (2 H, m, CH_2N), 3.28 (2 H, m, CH_2N), 3.60 (3 H, s, NMe), 3.69 (3 H, s, NMe); MS (EI) 310 (M), 173. The amine **7c** (800 mg, 2.6 mmol) was treated with phenylmethyl chloroformate (440 mg, 2.6 mmol) as for the synthesis of **6b**, except that the chromatographic eluant was $CHCl_3$/MeOH 10:1, to afford **6c** as white crystals (620 mg, 54%): mp 100-102 °C; IR 3360, 3280, 1700, 1640 cm^{-1}; NMR δ ($CDCl_3$) 1.29 [12 H, m, $(CH_2)_6$], 1.49 (2 H, m, CH_2), 1.65 (2 H, quintet, J = 6.8 Hz, CH_2), 3.08 (2 H, dt, J = 5.1, 7.1 Hz, CH_2), 3.18 (2 H, q, J = 6.6 Hz, NCH_2), 3.31 (3 H, s, NMe), 3.39 (3 H, s, NMe), 4.40 (1 H, br, NH), 4.81 (1 H, s, pyrimidine 5-H), 5.09 (2 H, s, $PhCH_2$), 7.35 (5 H, m, Ph-H_5); MS (EI) 444 (M), 336 (M − $PhCH_2OH$), 91 (100%). Anal. Calcd for $C_{24}H_{36}N_4O_4$: C, 64.82; H, 8.17; N, 12.61. Found: C, 65.1; H, 8.14; N, 12.65.

1,3-Dimethyl-6-[[[2-[2-(2-(phenylmethoxy)carbonyl]-amino]ethoxyethoxyethyl]amino]pyrimidine-2,4-dione (6d). The chloropyrimidine **5** (2.82 g, 16 mmol) was treated with 3,6-dioxaoctane-1,8-diamine (6.0 g, 40 mmol) as for the synthesis of **7b**, except that recrystallization was omitted, to give crude 1,3-dimethyl-6-[[2-[2-(2-aminoethoxy)ethoxy]ethyl]amino]pyrimidine-2,4-dione (**7d**) (3.03 g, 69%). This material was treated with phenylmethyl chloroformate (1.90 g, 11 mmol), as for the synthesis of **6b**, except that the chromatographic eluant was EtOAc — EtOAc/MeOH 6:1, to afford **6d** as a colorless gum (2.04 g, 44%): IR 3400–3280, 1740–1670 cm^{-1}; NMR δ ($CDCl_3$) 3.24 (2 H, q, J = 6 Hz, NCH_2), 3.31 (3 H, s, NMe), 3.36 (3 H, s, NMe), 3.40 (2 H, q, J = 5.3 Hz, NCH_2), 3.58 (2 H, t, J = 6 Hz, NCH_2CH_2), 3.62 (4 H, s, OCH_2CH_2O), 3.68 (2 H, t, J = 6 Hz, NCH_2CH_2), 4.83 (1 H, s, pyrimidine 5-H), 5.00 (1 H, br, NH), 5.09 (2 H, s, $PhCH_2$), 5.29 (1 H, br, NH), 7.35 (5 H, s, Ph-H_5); MS (EI) 421 (M), 91 (100%). Anal. Calcd for $C_{20}H_{28}N_4O_6$: C, 57.12; H, 6.72; N, 13.33. Found: C, 56.95; H, 6.78; N, 13.39.

1,3-Dimethyl-8-[[[5-(phenylmethoxy)carbonyl]amino]-pentyl]purine-2,6-dione (10a). Pentyl nitrite (410 mg, 3.5 mmol) was added carefully to **6a** (550 mg, 1.4 mmol) in EtOH (25 mL) at 40 °C, and the solution was stirred for 10 min. Hydrochloric acid (9 M, 0.3 mL) was added, and the mixture was stirred at ambient temperature for 16 h. The solvent was evaporated to afford crude **9a** as a red gum (430 mg, 73%). This material was boiled under reflux in BuOH (3 mL) for 45 min until decolorization had occurred. The evaporation residue was washed with Et_2O and recrystallized (BuOH) to give **10a** as off-white crystals (400 mg, 71% from **6a**): mp 182–184 °C; IR 3300, 1700, 1680, 1660 cm^{-1}; NMR δ 1.55 (2 H, quintet, J = 7.0 Hz, CH_2), 1.67 (2 H, quintet, J = 7 Hz, CH_2), 1.90 (2 H, quintet, J = 7.3 Hz, CH_2), 1.92 (2 H, t, J = 7.3 Hz, purine-CH_2), 3.23 (2 H, q, J = 6.4 Hz, CH_2N), 3.45 (3 H, s, NMe), 3.59 (3 H, s, NMe), 5.23 (2 H, br, $PhCH_2$), 7.54 (1 H, br, CbzNH), 7.58 (5 H, m, Ph-H_5); MS (EI) 400 (M), 292 (M − $PhCH_2OH$), 91 (100%). Anal. Calcd for $C_{20}H_{25}N_5O_4$: C, 60.14; H, 6.31; N, 17.53. Found: C, 60.09; H, 6.34; N, 17.50.

1,3-Dimethyl-8-[[[8-(phenylmethoxy)carbonyl]amino]-octyl]purine-2,6-dione (10b). The pyrimidine **6b** was treated with pentyl nitrite as for the synthesis of **9a**, to give crude **9b** as a purple gum. This material was heated, as for the synthesis of **10a**, to give **10b** as a cream solid (26%): mp 145–147 °C; IR 3320, 1740, 1680 cm^{-1}; NMR δ 1.24 (8 H, m, 4 × CH_2), 1.38 (2 H, quintet, J = 6 Hz, CH_2), 1.65 (2 H, quintet, J = 6 Hz, CH_2), 2.65 (2 H, t, J = 6.2 Hz, purine-CH_2), 2.95 (2 H, q, J = 6.2 Hz, NCH_2), 3.21 (3 H, s, NMe), 3.41 (3 H, s, NMe), 4.99 (2 H, s, $PhCH_2$), 7.22 (1 H, br, NH), 7.33 (5 H, m, Ph-H_5), 13.15 (1 H, s, NH); MS (EI) 442 (M), 334 (M − $PhCH_2OH$), 91 (100%). Anal. Calcd for $C_{23}H_{31}N_5O_4$: C, 62.57; H, 7.08; N, 15.86. Found: C, 62.99; H, 7.35; N, 15.79.

1,3-Dimethyl-8-[[[9-(phenylmethoxy)carbonyl]amino]-nonyl]purine-2,6-dione (10c). The 10-Cbz-aminodecylaminopyrimidine **6c** was treated with pentyl nitrite, as for the synthesis of **9a**, to afford crude **9c** as a red-purple oil. This material was boiled under reflux for 40 min in BuOH, and the solvent was evaporated. Recrystallization (BuOH) gave **10c** as pale buff crystals (300 mg, 45%): mp 95–96 °C; IR 3300, 1730, 1700, 1650 cm^{-1}; NMR δ 1.24 [10 H, m, $(CH_2)_5$], 1.38 (2 H, t, J = 6 Hz, CH_2), 1.67 (4 H, m, 2 × CH_2), 2.66 (2 H, q, J = 7.6 Hz, NCH_2), 2.97 (2 H, q, J = 6.4 Hz, purine-CH_2), 3.22 (3 H, s, NMe), 3.41 (3 H, s, NMe), 4.99 (2 H, s, $PhCH_2$), 7.22 (1 H, t, J = 6 Hz, NH), 7.34 (5 H, m, Ph-H_5); MS (EI) 455 (M), 347 (M − $PhCH_2OH$), 194 (100%). Anal. Calcd for $C_{24}H_{33}N_5O_4$: C, 63.26; H, 7.31; N, 15.38. Found: C, 63.6; H, 7.28; N, 15.4.

1,3-Dimethyl-8-[2-[2-(phenylmethoxy)carbonyl]ethoxy-ethoxymethyl]purine-2,6-dione (10d). The pyrimidine **6d** was treated with pentyl nitrite, as for the synthesis of **9a**, to give **9d** as a purple gum (97%). This material was heated as for the synthesis **10a**, to give **10d** as a yellow gum (66%): IR 3420, 3250, 1740, 1680 cm^{-1}; NMR δ 3.15 (2 H, q, J = 5.9 Hz, NCH_2), 3.23 (3 H, s, NMe), 3.40 (2 H, m, NCH_2CH_2), 3.42 (3 H, s, NMe), 3.55 (2 H, m) and 3.59 (2 H, m) (OCH_2CH_2O), 4.53 (2 H, s, purine-CH_2), 5.00 (2 H, s, $PhCH_2$), 7.33 (6 H, m, Ph-H_5 + NH), 13.57 (1 H, br, purine-NH); *m/z* (EI) 431 (M), 91 (100%). Anal. Calcd for $C_{20}H_{25}N_5O_6$: C, 55.68; H, 5.84; N, 16.23. Found: C, 55.40; H, 5.72; N, 16.39.

8-(5-Aminopentyl)-2,4-dimethylpurine-1,3-dione Hydrobromide (11a). The Cbz-protected amine **10a** (230 mg, 0.58 mmol) was stirred with HBr in AcOH (30%, 0.8 mL) for 15 min. Dry Et_2O (10 mL) was added, the suspension was stirred vigorously for 5 min, and the Et_2O was decanted. This washing procedure was repeated five times, and the solid was dried to afford **11a** as an off-white powder (200 mg, 100%): mp 250–252 °C; IR 3500–3400, 3240, 1740, 1680 cm^{-1}; NMR δ 1.31 (2 H, quintet, J = 6.7 Hz, CH_2), 1.56 (2 H, quintet, J = 7.3 Hz, CH_2), 1.70 (2 H, quintet, J = 7.3 Hz, CH_2), 2.70 (2 H, t, J = 7.7 Hz, purine-CH_2), 2.75 (2 H, t, J = 7.7 Hz, NCH_2), 3.22 (3 H, s, NMe), 3.41 (3 H, s, NMe), 7.74 (3 H, br, N^+H_3); MS (FAB) 266 (M + H), 180 (100%). Anal. Calcd for $C_{12}H_{20}BrN_5O_2$: C, 41.63; H, 5.82; N, 20.23. Found: C, 41.57; H, 5.77; N, 20.22.

8-(8-Aminooctyl)-2,4-dimethylpurine-1,3-dione Hydrobromide (11b). The Cbz-protected amine **10b** was treated with HBr, and the solid was washed with Et_2O, as for

the synthesis of **11a**, to give **11b** as a white powder (100%): mp 239–241 °C; IR 3440, 3200, 1760, 1680 cm^{-1}; NMR δ 1.27 [8 H, m, $(CH_2)_4$], 1.52 (2 H, m, CH_2), 1.67 (2 H, quintet, J = 6.7 Hz, CH_2), 2.68 (2 H, t, J = 7.6 Hz, purine-CH_2), 2.76 (2 H, sextet, J = 6.4 Hz, NCH_2), 3.22 (3 H, s, NMe), 3.41 (3 H, s, NMe), 7.72 (3 H, br, N^+H_3); MS (FAB) 308 (M + H), 180 (100%). Anal. Calcd for $C_{15}H_{26}BrN_5O_2$: C, 46.42; H, 6.75; N, 18.04. Found: C, 46.41; H, 6.67; N, 18.10.

8-(9-Aminononyl)-2,4-dimethylpurine-1,3-dione Hydrobromide (11c). The Cbz-protected amine **10c** was treated with HBr, and the solid was washed with Et_2O, as for the synthesis of **11a**, to give **11c** as an off-white powder (87%): mp 218–219 °C; IR 3160, 1720, 1680 cm^{-1}; NMR δ 1.25 (10 H, br, $CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2$), 1.51 (2 H, m, CH_2), 1.67 (2 H, m, CH_2), 2.68 (2 H, t, J = 7.5 Hz, purine-CH_2), 2.75 (2 H, m, NCH_2), 3.22 (3 H, s, NMe), 3.41 (3 H, s, NMe), 7.71 (3 H, s, N^+H_3); MS (FAB) 322 (M + H), 180 (100%). Anal. Calcd for $C_{16}H_{28}BrN_5O_2$: C, 47.77; H, 7.01; N, 17.41. Found: C, 47.84; H, 7.11; N, 17.22.

8-[2-(2-Aminoethoxy)ethoxymethyl]-2,4-dimethylpurine-1,3-dione Hydrobromide (11d). The Cbz-protected amine **10d** was treated with HBr, and the solid was washed with Et_2O, as for the synthesis of **11a**, to give **11d** as a pale yellow solid (100%): mp 220–221 °C; NMR δ 2.97 (2 H, sextet, J = 6 Hz, NCH_2), 3.23 (3 H, s, NMe), 3.43 (3 H, s, NMe), 3.62 (6 H, m, $OCH_2CH_2OCH_2$), 4.55 (2 H, s, purine-CH_2), 7.83 (3 H, br, N^+H_3); *m/z* (FAB) 298 (M + H), 180 (100%). Anal. Calcd for $C_{12}H_{20}N_5O_4Br$: C, 38.11; H, 5.33; N, 18.52. Found: C, 38.15; H, 5.27; N, 18.46.

5-[[N-[5-(2,4-Dimethyl-1,3-dioxopurin-8-yl)pentyl]thio]ureido]-3′,6′-dihydroxyspiro[isobenzofuran-1(3H),9′-(9H)xanthen]-3-one (13a). 3′,6′-Dihydroxy-5-isothiocyanatospiro[isobenzofuran-1(3*H*),9′-(9*H*)xanthen]-3-one (fluorescein-5-isothiocyanate; **12**) (340 mg, 870 μmol) was suspended in water (12 mL) and brought to pH 9.0 by dropwise addition of aqueous K_2CO_3 (1 M). To this stirred solution was added **11b** (300 mg, 870 μmol) in water (6.7 mL) and dioxane (3.3 mL) during 30 min. The mixture was stirred for 3 h. During this time, the pH was maintained at 9.0 ± 0.2 by dropwise addition of aqueous K_2CO_3 (1 M). The solution was acidified to pH 6.0 by dropwise addition of hydrochloric acid (2 M). Water (200 mL) was added, and the mixture was freeze-dried for 24 h to reveal an orange fluffy solid. Chromatography (EtOAc — EtOAc/MeOH 10:1) gave **13a** as an orange-yellow solid (280 mg, 50%): mp 197–198 °C; IR 3480, 1710, 1660 cm^{-1}; NMR δ 1.37 (2 H, m, CH_2), 1.62 (2 H, m, NCH_2CH_2), 1.76 (2 H, m, purine-CH_2CH_2), 2.73 (2 H, t, J = 7.7 Hz, purine-CH_2), 3.24 (3 H, s, NMe), 3.40 (3 H, s, NMe), 3.53 (2 H, m, NCH_2), 6.56 (2 H, dd, J = 8.8, 2.2 Hz, xanthene 2′,7′-H_2), 6.61 (2 H, d, J = 8.8 Hz, xanthene 1′,8′-H_2), 6.70 (2 H, d, J = 2.2 Hz, xanthene 4′,5′-H_2), 7.20 (1 H, d, J = 8.2 Hz, Ar 3-H), 7.73 (1 H, m, Ar 4-H), 8.25 (1 H, br, NH), 8.27 (1 H, br, Ar 6-H), 9.93 (1 H, br, NH), 10.13 (2 H, s, 2 × OH), 13.21 (1 H, br, purine NH); MS (FAB) 655 (M + H). Anal. Calcd for $C_{33}H_{30}N_6O_7S$: C, 60.53; H, 4.62; N, 12.84. Found: C, 60.61; H, 4.57; N, 12.72.

5-[[N-[8-(2,4-Dimethyl-1,3-dioxopurin-8-yl)octyl]thio]ureido]-3′,6′-dihydroxyspiro[isobenzofuran-1(3H),9′-(9H)xanthen]-3-one (13b). The 8-(8-aminooctyl)purine salt **11b** was treated with **12**, as for the synthesis of **13a**, to give **13b** as an orange-yellow solid (72%): mp 182–183 °C; IR 3500–3100, 1710, 1660 cm^{-1}; NMR δ 1.30 (8 H, m, 4 × CH_2), 1.55 (2 H, m, NCH_2CH_2), 1.66 (2 H, m, purine-CH_2CH_2), 2.67 (2 H, t, J = 7.3 Hz, purine-CH_2), 3.22 (3 H, s, NMe), 3.41 (3 H, s, NMe), 3.49 (2 H, m, NCH_2), 6.53 (2 H, dd, J = 8.8, 2.2 Hz, xanthene 2′,7′-H_2), 6.58 (2 H, d, J = 8.8 Hz, xanthene 1′,8′-H_2), 6.68 (2 H, d, J = 2.2 Hz, xanthene 4′,5′-H_2), 7.19 (1 H, d, J = 8.4 Hz, Ar 3-H), 7.70 (1 H, m, Ar 4-H), 8.27 (1 H, br, Ar 6-H), 10.23 (2 H, s, 2 × OH); *m/z* (FAB) 697 (M + H). Anal. Calcd for $C_{36}H_{36}N_6O_7S$: C, 62.05; H, 5.21; N, 12.01. Found: C, 62.11; H, 5.27; N, 12.02.

5-[[N-[9-(2,4-Dimethyl-1,3-dioxopurin-8-yl)nonyl]thio]ureido]-3′,6′-dihydroxyspiro[isobenzofuran-1(3H),9′-(9H)xanthen]-3-one (13c). The 8-(9-aminononyl)purine salt **11c** was treated with **12**, as for the synthesis of **13a**, to give **13c** as an orange-yellow solid (68%): mp 178–179 °C; IR 3400–3000, 1740, 1700, 1650 cm^{-1}; NMR δ 1.29 (10 H, br, 5 × CH_2), 1.56 (2 H, m, $NHCH_2CH_2$), 1.68 (2 H, m, purine-CH_2CH_2), 2.67 (2 H, t, J = 7.5 Hz, purine-CH_2), 3.22 (3 H, s, NMe), 3.41 (3 H, s, NMe), 3.48 (2 H, m, NCH_2), 6.56 (2 H, dd, J = 8.8, 2.2 Hz, xanthene 2′,7′-H_2), 6.59 (2 H, d, J = 8.8 Hz, xanthene 1′,8′-H_2), 6.67 (2 H, d, J = 2.2 Hz, xanthene 4′,5′-H_2), 7.17 (1 H, d, J = 8.4 Hz, Ar 3-H), 7.70 (1 H, m, Ar 4-H), 8.06 (1 H, br, CH_2NH), 8.22 (1 H, brs, Ar 6-H), 9.86 (1 H, br, Ar-NH), 10.18 (2 H, s, 2 × OH), 13.35 (1 H, purine NH); MS (FAB) 711 (M + H). Anal. Calcd for $C_{37}H_{38}N_6O_7S$: C, 62.52; H, 5.39; N, 11.82. Found: C, 62.45; H, 5.41; N, 11.75.

5-[N-[2-[2-(2,4-Dimethyl-1,3-dioxopurin-8-ylmethoxy)-ethoxyethyl]thio]ureido]-3′,6′-dihydroxyspiro[isobenzofuran-1(3H),9′-(9H)xanthen]-3-one (13d). The purine derivative salt **11d** was treated with **12**, as for the synthesis of **13a**, to give **13d** as an orange-yellow solid (77%): mp 1971–1998 °C; IR 3400–3200, 1740, 1700, 1650 cm^{-1}; δ 3.22 (3 H, s, NMe), 3.41 (3 H, s, NMe), 3.63 (8 H, m, $OCH_2CH_2OCH_2CH_2$), 4.54 (2 H, s, purine-CH_2), 6.56 (2 H, dd, J = 8.8 and 2.2 Hz, xanthene 2′,7′-H_2), 6.59 (2 H, d, J = 8.8 Hz, xanthene 1′,8′-H_2), 6.66 (2 H, d, J = 2.2 Hz, xanthene 4′,5′-H_2), 7.18 (1 H, d, J = 8.4 Hz, Ar 3-H), 7.73 (1 H, brd, J = 8.5 Hz, Ar 4-H), 8.1 (1 H, br, NH), 8.26 (1 H, br, Ar 6-H), 10.05 (1 H, br, NH), 10.14 (2 H, s, 2 × OH), 13.6 (1 H, purine NH); MS (FAB) 687 (M + H). Anal. Calcd for $C_{33}H_{30}N_6O_9S$: C, 57.71; H, 4.41; N, 12.24. Found: C, 57.61; H, 4.45; N, 12.22.

2,4-Dimethyl-8-[5-[5-(dimethylamino)naphthalene-1-sulfonylamino]pentyl]purine-1,3-dione Hydrobromide (14). The aminopentylpurine salt **11a** (200 mg, 580 μmol) in water (8 mL) and dioxane (4 mL) was brought to pH 9.5 by addition of aqueous Na_2CO_3 (0.5 M). 5-(Dimethylamino)naphthalene-1-sulfonyl chloride (160 mg, 580 μmol) in dioxane (10 mL) was added. The mixture was stirred for 3 h. During this time, the pH was maintained at 9.5–10.0 by dropwise addition of aqueous Na_2CO_3 (0.5 M). The solution was neutralized to pH 7.0 by addition of hydrochloric acid (2 M). Water (25 mL) was added and the mixture was freeze-dried for 24 h to reveal an orange fluffy solid. The residue, in ethyl acetate, was washed with water and dried. The solvent was evaporated, and the residue was recrystallized (EtOH) to give **14** as a green-yellow solid (160 mg, 55%): mp 183–185 °C; NMR δ 1.14 (2 H, quintet, J = 7 Hz, $CH_2CH_2CH_2CH_2CH_2$), 1.29 (2 H, quintet, J = 7 Hz, CH_2CH_2N), 1.46 (2 H, quintet, J = 7 Hz, purine-CH_2CH_2), 2.76 (2 H, t, J = 5.9 Hz, purine-CH_2), 2.81 (6 H, s, NMe_2), 3.22 (3 H, s, NMe), 3.39 (5 H, m, NMe + CH_2N), 7.24 (1 H, d, J = 7.3 Hz, naphthalene 6-H), 7.58 (1 H, t, J = 8.1 Hz, naphthalene 3-H or 6-H), 7.61 (1 H, d, J = 7.7 Hz, naphthalene 8-H), 7.87 (1 H, t, J = 7.9 Hz, naphthalene 6-H or 3-H), 8.08 (1 H, d, J = 7.3 Hz, naphthalene 4-H), 8.29 (1 H, d, J = 8.4 Hz, naphthalene 2-H), 13.1 (1 H, br, purine-NH); MS (FAB) 531.2020 (M + H) ($C_{24}H_{31}N_6O_4S$ requires 531.2026).

Immunoassay. The FCFD devices were fabricated according to the method of Badley *et al.* (7). The lower base plate was activated with (3-aminopropyl)trimeth-

Table 1. Binding Affinities of the Theophylline–Fluorescein Conjugates 13a–d to the Antibody Immobilized in the FCFD Device

conjugate	linker unit	binding affinity K_a (M^{-1})
theophylline (1)		$(0.06 \pm 0.02) \times 10^6$
13a	$(CH_2)_5$	$(3.1 \pm 1.5) \times 10^6$
13b	$(CH_2)_8$	$(1.4 \pm 0.6) \times 10^6$
13c	$(CH_2)_9$	$(3.2 \pm 1.8) \times 10^6$
13d	$CH_2O(CH_2)_2O(CH_2)_2$	$(1.0 \pm 0.4) \times 10^6$

oxysilane. Anti-theophylline IgA mouse monoclonal antibody was dissolved in HEPES buffer (0.1 M) containing NaCl (0.2 M) to a final concentration of 200 μg mL^{-1}. Aliquots of this solution (15 μL) were used for coupling of the antibody to the lower plate according to a carbonyldiimidazole method. The plates were then assembled in the usual way. Solutions of the theophylline–fluorescein conjugates **13a–d** were prepared in Tris-HCl buffer at pH 8.8. All assays using the FCFDs were performed in duplicate following incubation at ambient temperature for 20 min to ensure that the system had come fully to equilibrium. Total binding of **13a–d** to the antibody was measured by incubating the FCFDs with concentrations of conjugates from 0 to 5 μM. Nonspecific binding of **13a–d** to the antibody-loaded devices was measured in the presence of theophylline (1 mM). The specific binding isotherms were constructed by plotting (total fluorescence signal observed minus signal in the presence of excess theophylline) *vs* concentration of **13a–d**. From these plots (not shown) were obtained the binding affinities of each conjugate for the immobilized antibody (Table 1).

RESULTS AND DISCUSSION

Chemistry. Synthesis of 8-(2-aminoethyl)theophylline has been reported (*12*) involving acylation of 5,6-diamino-1,3-dimethylpyrimidine-2,4-dione (a relatively unstable compound) with *N*-protected 3-aminopropanoic acid, followed by cyclization under drastic conditions. We sought to develop a method that started with more readily available 1,ω-diamines and which did not use vigorous conditions for cyclization. Fuchs *et al.* (*12*) have suggested that treatment of 6-chloro-1,3-dimethylpyrimidine (**5**) with 1,ω-diamines would give the products of 1,ω-disubstitution. Thus, our initial approach involved setting up a synthon for hexane-1,6-diamine, which is protected at one amine only. Exchange of protecting groups on the commercially available mono-Boc hexane-1,6-diamine (**2**) was achieved by benzyloxycarbonylation at the free amine to give the orthogonally protected biscarbamate **3**, followed by acidolytic removal of Boc in excellent yield. The Cbz protection in **4** was designed to survive the reaction conditions in later steps, which would cleave Boc. Substitution of the chlorine of the 6-chloropyrimidinedione **5** with the amine **4** was effected in boiling 1,4-dioxane in the presence of inorganic base to give **6a** in satisfactory yield, as shown in Scheme 1. An attempt to raise the yield by use of refluxing ethanol as the reaction medium gave only a small amount of **6a** but encouraged substitution of chlorine by the solvent to give largely 1,3-dimethyl-6-ethoxypyrimidine-2,4-dione. Following the general method of Goldner *et al.* (*13*), nitrosation at C-5 of the pyrimidine was carried out with pentyl nitrite, in a reaction catalyzed by a small quantity of acid. Thermal cyclization/condensation of the brightly colored 5-nitrosopyrimidine **9a** to the Cbz-protected 8-(5-aminopentyl)theophylline **10a** was rapid and high-yielding in boiling butanol. Deprotection using hydrogen bromide in acetic acid was virtually quantitative in furnishing the 8-(5-aminopentyl)theophylline salt **11a**, which carries the required link and nucleophilic amine for attachment of electrophilically substituted fluorophores.

Selective monoprotection of longer chain alkane-1,ω-diamines, particularly 3,6-dioxaoctane-1,8-diamine, proved to be troublesome. However, by careful selection of reacting quantities and conditions, it proved possible to obtain good yields of the unprotected 6-[ω-amino(oxa)-alkylamino]pyrimidines **7b–d**, which could be separated with difficulty from traces of the products of 1,ω-disubstitution. Nevertheless, direct reaction of the crude product mixture with benzyl chloroformate gave the easily purified Cbz-protected 6-[ω-amino(oxa)alkylamino]-pyrimidines **6b–d**. As before, the sequence of nitrosation, thermal cyclization, and deprotection furnished the required 8-[ω-amino(oxa)alkylamino]theophylline salts **11b–d**.

The aminoalkyltheophyllines **11a–c** enabled study of the effect of the length of the link between hapten and fluorophore on the immunoreactivity of the final derivatives, whereas comparison of these with the diether linked compounds derived from **11d** indicated the importance of hydrophilicity of the link.

Fluorescein has absorption and fluorescence emission maxima at wavelengths that are appropriate for the FCFD device, with a large Stokes shift and a high quantum yield. The 5-isothiocyanate or mixtures of the 4- and 5-isothiocyanates have frequently been used for fluorescent labeling of amino groups of biological molecules in aqueous media (*15*, *16*). Fluorescein also confers aqueous solubility on its derivatives, a factor important for the target fluorescent theophylline derivatives. To have a chemically defined system, the 5-isothiocyanate was used to thiocarbamoylate the amines **11a–d**. It was found necessary to control closely the pH of the aqueous reaction mixture in the range 9.0 ± 0.2 to ensure that the amine was not protonated and that the isothiocyanate was not subject to base-catalyzed hydrolysis. To provide an alternative fluorescent derivative of theophylline for our studies with different wavelength maxima for excitation and fluorescence, 8-(5-aminopentyl)theophylline (**11a**) was also coupled with dansyl chloride [5-(dimethylamino)naphthalene-1-sulfonyl chloride] under aqueous basic conditions, although this reaction tolerated a higher pH (9.5–10.0), owing to the lower lability of dansyl chloride to hydrolysis. The dansylaminopentyltheophylline **14** was formed in good yield.

The ^{1}H NMR spectra of the fluorescein–theophylline derivatives **13a–d** were examined in solution in deuteriodimethyl sulfoxide. The signals for the xanthene moiety show considerable symmetry, in which H-1′ and H-8′ are magnetically equivalent, as are H-2′ and H-7′ and H-4′ and H-5′, respectively. Most interestingly, the phenolic OH signal appears as a 2 H singlet at δ 10.2, confirming that the fluorescein is present in this solvent as the tautomer shown, rather than the lactone-opened carboxylate tautomer.

Straightforward syntheses of a range of 8-[ω-amino-(oxa)alkyl]theophyllines have been achieved, providing convenient access to fluorescent derivatives with applications in automated immunoassays for theophylline.

Immunoassay. The initial requirement of the study was to determine the antibody-binding affinities of the fluorescent theophylline conjugates **13a–d**, and it was sensible to carry out these experiments on the immobilized antibody within the FCFD by introducing the conjugates in solution to the FCFD in the presence and absence of excess theophylline. The FCFD reader was able to determine the relative intensity of surface fluo-

rescence, and the antibody-bound fluorescence was determined by subtracting the nonspecific fluorescence, in the presence of excess theophylline, from the total fluorescence. Initial experiments were conducted to establish the equilibration time of the system, by recording the total fluorescence at 30 s intervals over a 30 min period. Binding equilibrium was achieved within approximately 10 min, and a standard incubation time of 20 min was adopted for further studies. Specifically bound fluorescence was plotted against concentration of each conjugate, according to the Langmuir equation, to allow determination of the binding affinities of **13a–d** (Table 1). The binding affinity of theophylline could then be determined by competition with a fluorescent derivative, in this case with **13a** (Table 1). The binding affinities of the conjugates compared well with that of theophylline, indicating that this antibody could tolerate substitution of theophylline at position 8. The affinities were almost independent of the length [$(CH_2)_5$ *vs* $(CH_2)_9$] or the hydrophobic/hydrophilic nature of the link [$(CH_2)_9$ *vs* $CH_2O(CH_2)_2O(CH_2)_2$] between the theophylline hapten and the fluorophore. Compound **13a** was identified by a small margin as being the conjugate of choice and was selected for further testing and development of assay procedures. Briefly, it was established that the fluorescent conjugates had the appropriate antibody-binding properties for development of an FCFD-based assay for theophylline, within a concentration range which would be useful for clinical use (0–300 μM theophylline). Compound **13a** was used at a concentration of 10^{-6} M within the FCFD device, with low levels of interference from nonspecific surface-bound fluorescence. Theophylline competed with **13a** such that the signal recorded by the FCFD reader decreased from maximum to minimum levels between theophylline concentrations of 5 and 500 μM. Precision was determined at theophylline concentrations of 25, 50, and 125 μM, the coefficients of variation being 6.5%, 6.0%, and 5.2% (within-assay) and 8.0%, 7.5%, and 5.0% (between-assay), respectively. This concentration range corresponded to a useful clinical range, although the precision of the assay in estimating the concentration of theophylline was compromised by insufficient total fluorescence. This total fluorescence could be increased by immobilizing a higher mass of antibody to the bottom plate of the FCFD. Nevertheless, the FCFD devices studied here would be capable of distinguishing between low (5 μM) and high (250 μM) clinical levels of theophylline, which could be useful in acute conditions to establish whether a patient is presenting with overdose or underdose of theophylline.

Preliminary experiments were carried out to test the cross-reactivity of the system with other xanthines, including caffeine, theobromine, and metabolites of theophylline. Initial results suggested that the concentration of each xanthine required to displace **13a** was at least 100 times greater than that for theophylline. Thus, little interference can be expected from other blood-borne xanthines. It was also established that the assay worked as well in the presence of 5 mg mL^{-1} human serum albumin, with a slight loss of sensitivity presumably due to interactions between xanthines and albumin, which reduced the effective concentration of free xanthines in the FCFD.

The results of further studies on the development and validation of the immunoassays will be reported in full elsewhere.

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Biologically Compatible Linear Block Copolymers of
Polyalkylene Oxide and Peptide Units

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[Patent]

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[54] **BIOLOGICALLY COMPATIBLE LINEAR BLOCK COPOLYMERS OF POLYALKYLENE OXIDE AND PEPTIDE UNITS**

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[58] Field of Search 424/78.38, 78.3, 424/78.17; 525/54.1, 423, 430

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[57] ABSTRACT

A linear block copolymer comprising units of an alkylene oxide, linked to units of peptide via a linking group comprising a —$CH_2CHOHCH_2N(R)$— moiety, is useful as an imaging agent, drug, prodrug or as a delivery system for imaging agents, drugs or prodrugs.

17 Claims, No Drawings

BIOLOGICALLY COMPATIBLE LINEAR BLOCK COPOLYMERS OF POLYALKYLENE OXIDE AND PEPTIDE UNITS

BACKGROUND OF THE INVENTION

a) Field of the Invention

This invention relates to block copolymers useful in diagnostic imaging, drug delivery, and as drugs.

b) Information Disclosure Statement

Nathen et al, Bioconjugate Chemistry 4 54–62 (1993) discloses copolymers of lysine and polyethylene glycol prepared by reacting amino groups of lysine with activated ester derivatives of polyethylene glycol. The polymer is best described as a polyamide formed by ϵ-amino and the α-amino of lysine.

Davis et al., U.S. Pat. No. 4,179,337 dated Dec. 18, 1979 discloses insulin coupled to polyethylene glycol or polypropylene glycol having a molecular weight of 500 to 20,000 molecular weight.

Zilkha et al, U.S. Pat. No. 3,441,526 issued Apr. 29, 1969 discloses N-carboxyanhydride-based polymerization of polypeptide with polyethylene glycol, no examples of the preparation are given.

British Patent 1,469,472 discloses low molecular weight polyethylene oxide immobilized proteins, said to have low immunogenicity.

However, none of these references suggests a linear block copolymer having repeating units of an alkylene oxide linked to repeating units of a peptide through a linking group formed by the reaction of an amine precursor and an epoxide precursor. Moreover, the prior art teaches that crosslinking (via amino acid side chains) often frustrates the linear copolymerization often sought. The invention described herein advantageously avoids such crosslinking.

SUMMARY OF THE INVENTION

The invention concerns a linear block copolymer comprising single or repeating units of poly(alkylene oxide) (PAG) linked to units of peptide. The copolymer can be tailored to produce water-soluble polymers which are stable in the blood circulation but ultimately will be degraded to allow more facile excretion of low molecular weight PAG derivatives in the urine.

In one aspect of the invention, the copolymer is a diagnostic agent, and/or a therapeutic agent and/or a targeting agent.

In another aspect of the invention, the copolymer is a chelating copolymer, having drug, and/or a prodrug and/or a chelating moiety attached to side chains of the peptide. Such moieties are useful in chelating metals, especially metals useful in diagnostic imaging of a body or tissues or as cytotoxic agents.

In another aspect the invention is a targeting delivery system for drugs and prodrugs.

In yet another aspect of the invention, the invention comprises the reaction of an epoxide and an amine to produce a novel polymer.

DETAILED DESCRIPTION INCLUSIVE OF PREFERRED EMBODIMENTS

We have discovered that linear block copolymers comprising blocks of poly(alkylene oxide) (PAGs) and peptides attached via —$CH_2CHOHCH_2N(R)$— based linking groups

are useful as imaging agents, prodrugs, drugs and drug delivery systems. Preferred copolymers are of the formula;

—(PAG)N(R)$CH_2CHOHCH_2OC_6H_4CO$(Peptide)$NH(CH_2)_pC_6H_4$ $OCH_2CHOHCH_2N(R)$— Formula A

or

—(PAG)$CH_2CHOHCH_2N(R)CH_2CO$(Peptide)$NH(CH_2)_pNHCO$ $CH_2NR\ CH_2CHOHCH_2$— Formula B

wherein

R is a 1–4 carbon alkyl; and

p is from 1 to 6 and the peptide is preferably Gly-Phe-Leu-Gly or Lys-Gly-Phe-Leu-Gly.

The compounds can be tailored for specific uses by altering the size of the polymer or altering the peptide composition to provide differing blood pool residence time, enzymatic breakdown rates, and tissue distributions.

As an imaging agent, said composition preferably has a molecular weight of at least about 5000 and a metal ion useful as a contrast enhancer, fluorophore or x-ray opaque ion associated therewith, and thus suitable for use as an agent for diagnostic imaging.

An imaging metal is defined as a metal useful in x-ray imaging or a metal useful in magnetic resonance imaging, preferably a paramagnetic metal and more preferably a lanthanide metal or transition metal; or a metal useful in fluorescence imaging, preferably a lanthanide metal, most preferably Europium.

This invention further provides a method of performing a diagnostic imaging procedure in a body comprising administering to the body a contrast enhancing amount of the polymer described above, and then exposing the body to a magnetic resonance measurement step to image at least a portion of the body.

It is a particularly advantageous feature that the polymeric chelates of this invention provide effective imaging contrast enhancement of the blood pool within the vascular system for remarkably long periods of time.

It is another advantageous feature of this invention that polymeric compounds are provided having a specificity toward accumulation in different tissues, for example, in tumors and the liver.

As used herein, PAG refers to poly alkylene oxide moieties having a single type of repeating unit or differing (non-repeating) units of alkylene oxide, or a mixture thereof in each PAG. Each alkylene oxide unit in the PAG contains from 2 to about 4 carbons, linear or branched. Poly(alkylene oxide) units in the polymer may also differ in length and composition from each other. Exemplary PAG moieties include poly(ethylene oxides), poly(propylene oxides) and poly(butylene oxides). Preferred PAG moieties include poly(ethylene oxides), poly(propylene oxides) and random and block copolymers thereof. Poly(ethylene oxide)-containing polymers are particularly preferred when it is desired for the final polymer to possess solubility in water. It is also contemplated that the poly(alkylene oxide) moiety can comprise glycerol poly(alkylene oxide) triethers, polyglycidols, linear, block and graft copolymers of alkylene oxides with compatible comonomers such as poly(proplene oxide-co-ethylene oxide), or poly(butylene oxide-co-ethylene oxide) and grafted block copolymers. These moieties can be derived from poly(alkylene oxide) moieties which are commercially available or alternatively they can be prepared by techniques well known to those skilled in the art. A particularly preferred class of polyalkyleneoxide moieties derived from poly(ethylene oxide) can be represented by the structure:

$-O(CH_2CH_2O)_mCH_2CH_2O-$

wherein m is 1 to 750. The preferred length depends upon the desired molecular weight.

These PAG moieties and their reactive derivatives, useful in preparing the polymer of the invention, are known in the art. For example, bis(methyl amino) polyethylene glycol and its use as an intermediate in the preparation of block copolymers is known in the art, for example; Mutter, Tetrahedron Letters, 31, 2839–2842 (1978) describes a procedure to convert the terminal hydroxyl groups of poly(ethyleneoxide) to reactive primary amino groups as well as the preparation of a number of reagents bound to poly(ethyleneoxide) amines; Harris et al, J. Polymer. Science, 22, 341–352 (1984) describe various PAG derivatives including for example, amino poly(ethyleneoxide). Other PAG derivatives are prepared by known chemistries examples of which are described hereinbelow.

As used herein, peptide refers to an amino acid chain of at least 2 amino acids, wherein each of the amino acids in the peptide may or may not be all the same, and may or may not all be selected from the 20 naturally occurring L-amino acids, but contain D-amino acids, artificial amino acids or amino acid derivatives, such as glutamate esters, lysyl(ε-amino)amides and the like. This definition also includes proteins, polypeptides and oligopeptides, which are art recognized amino acid chains. Specifically contemplated preferred peptides include small enzymes (less than 100 kd), peptide hormones, peptide recognition domains, peptide drugs, and peptides with known enzymatic breakdown rates.

Certain abbreviations appearing in the text and schemes are here defined: Boc refers to the art recognized t-butoxy carbonyl radical commonly used as a blocking agent in solid phase peptide synthesis. Conventional three letter abbreviations for amino acid residues are used throughout the specification. OPFP refers to pentafluorophenyloxy; Bn refers to benzyl; CBZ refers to phenylmethoxycarbonyl; OTCP refers to 2,4,5-trichlorophenyloxy; Troc refers to 2,2,2-trichloroethoxycarbonyl.

Copolymerization can occur by reaction of bis(oxiranyl-)derivatives (also known as bis(epoxides)) with bis(amino or alkylamino) derivatives (also known as bis amines). There are no by-products of the polymerization reaction. The monomer units of PAG and peptide can be prepared as either bis(oxiranyl) derivatives or bis(amino) derivatives provided that the reaction producing the copolymers is between an amine and an epoxide. Therefore there are two chemical strategies for preparing products of the invention described hereinbelow. As a consequence of reacting bisamines with bisepoxides the sense of the PAG and peptide units can be reversed.

The polymer of the invention has between its PAG and peptide subunits, a linking group. The linking group contains a $-CH_2CHOHCH_2N(R)-$ diradical, typically derived from the reaction of an amine and an epoxide. It is preferred that a bisepoxide subunit be reacted with a bisamine subunit. The skilled artisan will appreciate that the recitation used throughout the specification of each type of linking group diradical can be reversed and have the same meaning. Thus the sense of the linking group can be reversed (end for end), with one terminus attached to the PAG moiety, and the other terminus attached to the peptide or vise versa, while its recitation in the specification and the claims is the same.

Examples of suitable linking groups include

$-CONH(CH_2)_pNHCOCH_2N(CH_3)CH_2CHOHCH_2OC_6H_4-$;

$-CONH(CH_2)_pNHCOCH_2N(CH_3)CH_2CHOHCH_2OC_6H_4CO-$;

$-CONH(CH_2)_pNHCOCH_2N(CH_3)CH_2CHOHCH_2OC_6H_4(CH_2)_2-$;

$-CONH(CH_2)_pNHCOCH_2N(CH_3)CH_2CHOHCH_2OC_6H_4(CH_2)_2NH-$;

$-CONH(CH_2)_pNHCOCH_2N(CH_3)CH_2CHOHCH_2-$;

$-CONH(CH_2)_pNHCOCH_2N(CH_3)CH_2CHOHCH_2OC_6H_4(CH_2)_2-$;

$-CONH(CH_2)_pNHCOCH_2N(CH_3)CH_2CHOHCH_2OC_6H_4(CH_2)_2NH-$;

$-NH(CH_2)_pN(CH_3)CH_2CHOHCH_2OC_6H_4-$;

$-NH(CH_2)_pN(CH_3)CH_2CHOHCH_2OC_6H_4CO-$;

$-NH(CH_2)_pN(CH_3)CH_2CHOHCH_2OC_6H_4(CH_2)_2-$;

$-NH(CH_2)_pN(CH_3)CH_2CHOHCH_2OC_6H_4(CH_2)_2NH-$;

$-CONH(CH_2)_pNHCO(CH_2)pN(CH_3)CH_2CHOHCH_2-$;

$-NH(CH_2)_pNHCO(CH_2)pN(CH_3)CH_2CHOHCH_2-$;

$-NHCO(CH_2)_pN(CH_3)CH_2CHOHCH_2-$; and

$-CO(CH_2)_pN(CH_3)CH_2CHOHCH_2-$.

Peptides used to prepare the invention can be prepared by standard procedures known in the art. Useful peptides include those derived from native or recombinant organisms, solid phase peptide synthesis or traditional wet chemistry peptide synthesis and the like. Each of these peptide preparation methods are well known in the art and use conventional, known materials. Protein expression and purification from natural and recombinant sources is in the prior art (cf. *Protein Expression and Purification* (1990); Harris et al., *Protein Purification Methods* (1989); Deutscher, M. P. *Guide to Protein Purification Methods in Enzymology*, Vol. 82 (1990)). Peptide synthesis is also known in the art (cf. Atherton, et al., *Solid Phase Peptide Synthesis a Practical Approach*, Oxford University Press (1989)). Thus, the peptides are easily prepared by known chemistry.

Linear peptide fragments can be tailored such that they are stable in blood, but are susceptible to lysosomal degradation by commonly occurring proteases. Examples of susceptible peptide units are gly-phe-leu-gly, gly-phe-tyr-ala, ala-gly-val-phe, gly-phe-ala-gly, and others known in the art. The prior art describes such oligopeptides as useful in preparing prodrugs, when the drug is attached to one terminus of the oligopeptide. (See generally "Polymers Containing Enzymatically Degradable Bonds" *Makromol. Chem.* 184 (1983) R. Duncan, H. C. Cable, J. B. Lloyd, P. Rejmanov'a and J. Kopecek, in Polymers containing enzymatically degradable bonds, 7. Design of oligopeptide sidechains to promote efficient degradation by lysosomal enzymes, *Makromol. Chem.*, 184, p. 1997–2008 (1983); and P. Rejmanova, J. Kopecek, J. Pohl, M. Baudys and V. Kostva, in Polymers containing enzymatically degradable bonds. 8. Degradation of oligopeptide sequences in N-(2-hydroxypropyl)methacrylamide copolymers by bovine spleen cathepsin B, *Makromol. Chem.* 184, p. 2009–2020, (1983).) In this invention it is contemplated that prodrugs can be attached to functionalized side chains of the peptide, rather than the terminus of the peptide.

The concept of drug targeting has gained importance in recent years, especially for anticancer drugs, inasmuch as toxic side effects of anticancer drugs to normal cells are a primary obstacle in cancer chemotherapy due to lack of selectivity of the anticancer drugs to cancer cells. In the prior art, drug targeting has been accomplished by drug conjugation with large antibodies, or encapsulation in a transporter specific to the target. Materials such as proteins, saccharides, lipids and synthetic polymers have been used for such transporters. Antibodies have been perhaps most widely used due to their target specificity and wide applicability. However, these methodologies have not been commercially exploited because the prohibitive cost of the transporter or targeting agent which can be used to target only one type of cell or tissue.

The peptide portion of the polymer can be tailored to recognize (or target) certain cells or functions of cells. Because the polymer can use more than one peptide and thus more than one type of peptide, the polymer can advantageously target more than one type of cell or tissue at once. Judicious choice of peptide allows treatment or targeting of

more than one type of cancer cell, for example, or other disease state. This choice is facilitated by the prior art which contains a myriad of known oligopeptides which are antigenic to certain cells. Furthermore, the invention allows such targeting without the cost of raising antibodies to certain cells, harvesting such antibodies, conjugating antibodies to drug and further testing for maintained specificity after conjugation. The invention allows specific targeting to be achieved by short recognition sequences. Cell specific delivery can be achieved by incorporating targeting agents into the polymer. Preferred peptides are those which have a receptor molecule specific to a ligand of interest. Thus, a specific binding reaction involving the reagent can be used for the targeting expected.

Depending upon the intended use, the peptides can be selected from a wide variety of naturally occurring or synthetically prepared materials, including, for example enzymes, proteins, peptide hormones, virus coats, or proteins derived from blood components, tissue and organ components, including haptens, antibodies, antigenic proteinaceous materials, or fragments of any of these and others known to one skilled in the art.

Examples of these targeting peptides include: the integrin binding motif RGDS (arg-gly-asp-ser), which is present on many extracellular matrix proteins and can be used to interfere with cell adhesions involved in migration of leukocytes. Other peptide sequences which can be used to deliver the polymer include cationic sequences (ie. rich in lys or arg) which are useful in producing a DNA-binding polymer for use in supression of gene expression, antisense oligomer delivery and the like; peptide hormones such as αMSH which can be used for targeting to melanoma; and relatively low molecular weight (15–20 kDa) engineered hypervariable antibody binding domains (V_H+V_L constructs) raised against any target. Such sequences are obtained by synthesis, isolation from cells or bacteriophages or they can also be raised against cells, proteins, or foreign substances in a host. Common hosts for raising recognition sequences include rabbits, goats, mice, and the like. These and other methods of obtaining recognition sequences are known in the art.

In certain embodiments, the above-described peptide can be an immunoreactive group, which would be found in a living organism or which finds utility in the diagnosis, treatment or genetic engineering of cellular material of living organisms. The peptide has a capacity for interaction with another component which may be found in biological fluids, cells or associated with cells to be treated or imaged, such as, for example tumor cells and the like.

Two highly preferred uses for the polymer of this invention are for the diagnostic imaging of tumors and the treatment of tumors. Preferred immunoreactive groups therefore include antibodies, or immunoreactive fragments thereof, to tumor-associated antigens. Specific examples include B72.3 antibodies (described in U.S. Pat. Nos. 4,522,918 and 4,612,282) which recognize colorectal tumors, 9.2.27 anti-melanoma antibodies, D612 antibodies which recognize colorectal tumors, UJ13A antibodies which recognize small cell lung carcinomas, NRLU-10 antibodies which recognize small cell lung carcinomas and colorectal tumors (Pan-carcinoma), 7E11C5 antibodies which recognize prostate tumors, CC49 antibodies which recognize colorectal tumors, TNT antibodies which recognize necrotic tissue, PR1A3 antibodies, which recognize colon carcinoma, ING-1 antibodies, which are known in the art and are described in International Patent Publication WO-A-90/02569, B174 antibodies which recognize squamous cell carcinomas, B43 antibodies which are reactive with certain lymphomas and leukemias and any other antibody which may be of particular interest.

Because the peptides of the polymer are linear, they can provide functional groups for coupling of diagnostic agents, drugs, or prodrugs or other targeting moieties by the side chains of individual amino acids found in the peptide portion of the backbone. Functional groups can also be added by reacting or derivatizing functionalizable basic groups (found for example in lysyl or arginiinyl residues) or acidic groups (as found in aspartate, glutamate, providing free carboxyl groups), or sulfhydryl groups, (e.g. cysteine), hydroxyl groups (such as found in serine) and the like. This coupling is done by standard peptide chemistry known in the art.

Cytotoxic drugs can also be coupled to the polymer to produce prodrugs which are released as a drug to targeted cells or tissues. Such coupling methods are known in the art, see for example; Duncan, P. Kopeckova-Rejmanova, J. Strohalm, I. Hume, H. C. Cable, J. Pohl, J. B. Lloyd and J. Kopecek (1987) Anti-cancer agents coupled to N-(2-hydroxypropyl)methacrylamide copolymers. I. Evaluation of daunomycin and puromycin conjugates in vitro. British J. Cancer, 55:165–174. R. Duncan, P. Kopeckova, J. Strohalm, I. Hume, J. B. Lloyd and J. Kopecek (1988) Anti-cancer agents coupled to N-(2-hydroxypropyl)methacrylamide copolymers. II. Evaluation of daunomycin conjugates in vivo against L1210 leukaemia. British J. Cancer, 57:147–156. Drugs contemplated to be useful include any drug which can be covalently attached to the polymer and retains its activity when so attached. It is contemplated that drugs which become active only when liberated from the polymer are also useful, and as such are prodrugs.

Drugs which are contemplated to be useful in the polymer include cytotoxic agents, and immunomodulating peptides and proteins as described above.

By "cytotoxic agent", it is meant any agent able to kill cells, including, chemotherapeutic agents such as cytotoxic drugs and cytotoxic antibiotics, chelated radionuclides and toxins or any agent which initiates or which leads to cell death. The term cytotoxic agents also includes agents which activate a host's immune response leading to cell death. The cytotoxic agent will be selected with reference to factors, such as the type of disease state, for example the type of cancer tumor and the efficacy of a certain chemotherapy agent for treating the cancer tumor involved, and the like. The cytotoxic agent may be selected from alkylating agents, antimetabolites, natural products useful as cytotoxic drugs, hormones and antagonists and other types of cytotoxic compounds.

Examples of alkylating agents include the nitrogen mustards (i.e. the 2-chloroethylamines) such as, for example, chloromethine, chlorambucil, melphalan, uramustine, mannomustine, extramustine phosphate, mechlor-thaminoxide, cyclophosphamide, ifosamide and trifosfamide; alkylating agents having a substituted aziridine group such as, for example, tretamine, thiotepa, triaziquone and mitomycin; alkylating agents of the alkyl sulfonate type, such as, for example, busulfan and piposulfan; alkylating N-alkyl-N-nitrosourea derivatives such as, for example, carmustine, lomustine, semustine or streptozotocine; alkylating agents of the mitobronitole, dacarbazine and procarbazine type; and platinum complexes such as, for example, cisplatin and carboplatin and others.

Examples of antimetabolites include folic acid derivatives such as, for example, methotrexate, aminopterin and 3'-dichloromethotrexate; pyrimidine derivatives such as, for example, 5-fluorouracil, floxuridine, tegafur, cytarabine, idoxuridine, and flucytosine; purine derivatives such as, for example, mercaptopurine, thioguanine, azathioprine, tiamiprine, vidarabine, pentostatin and puromycin and others.

Examples of natural products, useful as cytotoxic agents include for example vinca alkaloids, such as vinblastine and vincristine; epipodophylotoxins such as, for example, etoposide, and teniposide; antibiotics such as, for example, adrimycin, daunomycin, dactinomycin, damorubicin, doxorubicin, mithramycin, bleomycin and mitomycin; enzymes such as, for example, L-asparaginase; biological response modifiers such as, for example, alpha-interferon; camptothecin; taxol; and retinoids such as retinoic acid and the like.

Examples of hormones and antagonists include adrenocorticoids, such as, for example, prednisone; progestins, such as, for example, hydroxyprogesterone acetate, medroxyprogesterone acetate and megestrol acetate; estrogens such as, for example, diethylstilbestrol and ethinyl estradiol; antiestrogens such as for example, tamoxifen; androgens such as, for example, testosterone propionate and fluoxymestrone; antiandrogens such as, for example, flutamide; and gonadotropin-releasing hormone analogs such as, for example, leuprolide.

Examples of miscellaneous cytotoxic agents include anthracenediones such as for example, mitoxantrone; substituted ureas such as, for example, hydroxyureas; and adrenocortical suppressants such as, for example, mitotane and aminoglutethimide. The cytotoxic agent can be ionically associated with the chelating residue. For example, in preferred embodiments, the cytotoxic agent is a radionuclide comprising a radioactive metal ion such as described below associated with a peptide-linked chelating residue. The polymer of the invention can contain one or more of a wide variety of chelating agents. As is well known, a chelating agent is a compound containing donor atoms that can combine by coordinate bonding with a cation to form a cyclic structure called a chelation complex or chelate. This class of compounds is described in the Kirk-Othmer Encyclopedia of Chemical Technology, Vol. 5, 339 –368.

Chelating residues may also be attached via the functionalizable side chains of the peptide via known chemistry. These chelating residues can be coupled to the polymer to produce contrast agents useful in diagnostic imaging or cytotoxic agents when complexed with the appropriate metal. The chelating residue is attached to an available amino acid side chain in the peptide portion of the polymer by a protein reacting group. By "protein reactive group" it is meant any group which can react with any functional groups typically found in proteins, especially an amino acid side chain.

Preferred protein reactive groups can be selected from but are not limited to:

(1) A group that will react directly with the amine or sulfhydryl groups on an amino acid side chain. For example, active halogen containing groups including, for example, chloromethylphenyl groups and chloroacetyl [Cl—CH_2CO—] groups, activated 2-leaving-group-substituted ethylsulfonyl and ethylcarbonyl groups such as 2-chloroethylsulfonyl and 2-chloroethylcarbonyl; vinylsulfonyl; vinylcarbonyl; epoxy; isocyanato; isothiocyanato; aldehyde; aziridine; succinimidoxycarbonyl; activated acyl groups such as carboxylic acid halides; mixed anhydrides and the like; and other groups known to be useful in attaching molecules to proteins or crosslinking proteins and the like.

(2) A group that can react readily with modified proteins or similar biological molecules modified to contain reactive groups such as those mentioned in (1) above, for example, by oxidation of the amino acid side chain to an aldehyde or a carboxylic acid, in which case the "protein reactive group" can be selected from amino, alkylamino, arylamino, hydrazino, alkylhydrazino, arylhydrazino, carbazido, semicarbazido, thiocarbazido, thiosemicarbazido, sulfhydryl, sulfhydrylalkyl, sulfhydrylaryl, hydroxy, carboxy, carboxyalkyl and carboxyaryl. The alkyl portions of the protein reactive group can contain from 1 to about 18 carbon atoms as described for R above. The aryl portions of the protein reactive group can contain from about 6 to about 20 carbon atoms.

(3) A group that can be linked to the amino acid side chain or similar biological molecule, or to the modified peptide as noted in (1) and (2) above by use of a crosslinking agent. Certain useful crosslinking agents, such as, for example, difunctional gelatin hardeners, bisisocyanates etc., which become a part of a linking group in the polymer during the crosslinking reaction. Other useful crosslinking agents, such as, for example, consumable catalysts, are not present in the final conjugate. Examples of such crosslinking agents are carbodiimide and carbamoylonium crosslinking agents as disclosed in U.S. Pat. No. 4,421,847 and the dication ethers of U.S. Pat. No. 4,877,724. With these crosslinking agents, one of the reactants must have a carboxyl group and the other an amine, alcohol, or sulfhydryl group. The crosslinking agent first reacts selectively with the carboxyl group, then is cleaved during reaction of the "activated" carboxyl group with, for example, an amine to form an amide linkage between the peptide portion of the polymer and metal complexing agents, thus covalently bonding the two moieties. An advantage of this approach is that crosslinking of like molecules, e.g., amino acid side chains with amino acid side chains or complexing agents with complexing agents is avoided, whereas the reaction of difunctional crosslinking agents is less selective. Especially preferred protein reactive groups include amino and isothiocyanato. Preferred chelating agent precursors have anhydride, sulfonylylchloride, alkylsulfate, vinyl sulfate, or ester functionalkyl.

The chelating residues can be derived from chelating moieties which are selected to contain electron donating atoms which will chelate a metal, by forming coordination bonds therewith. These moieties can be selected from polyphosphates, such as sodium tripolyphosphate and hexametaphosphoric acid;

aminocarboxylic acids, such as ethylenediaminetetra-acetic acid, N-(2-hydroxyethyl)ethylenediaminetriacetic acid, nitrilotriacetic acid, N,N-di(2-hydroxyethyl)glycine, ethylenebis(hydroxyphenylglycine) and diethylenetriamine pentaacetic acid;

1,3-diketones, such as acetylacetone, trifluoroacetylacetone, and thenoyltrifluoroacetone;

hydroxycarboxylic acids, such as tartaric acid, citric acid, gluconic acid, and 5-sulfosalicylic acid;

polyamines, such as ethylenediamine, diethylenetriamine, triethylenetetramine, and triaminotriethylamine;

aminoalcohols, such as triethanolamine and N-(2-hydroxyethyl)ethylenediamine;

aromatic heterocyclic bases, such as 2,2'-dipyridyl, 2,2'-diimidazole, dipicoline amine and 1,10-phenanthroline;

phenols, such as salicylaldehyde, disulfopyrocatechol, and chromotropic acid;

aminophenols, such as 8-hydroxyquinoline and oxinesulfonic acid;

oximes, such as dimethylglyoxime and salicylaldoxime;

peptides containing proximal chelating functionality such as polycysteine, polyhistidine, polyaspartic acid, polyglutamic acid, or combinations of such amino acids;

Schiff bases, such as disalicylaldehyde 1,2-propylenediimine;

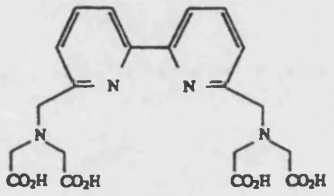
tetrapyrroles, such as tetraphenylporphin and phthalocyanine;

sulfur compounds, such as toluenedithiol, meso-2,3-dimercaptosuccinic acid, dimercaptopropanol, thioglycolic acid, potassium ethyl xanthate, sodium diethyldithiocarbamate, dithizone, diethyl dithiophosphoric acid, and thiourea;

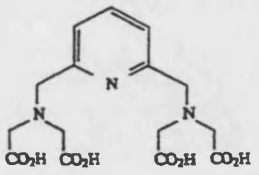
synthetic macrocylic compounds, such as dibenzo[18] crown-6, $(CH_3)_6$-[14-]4,11-diene-N_4, and (2.2.2)-cryptate; and

phosphonic acids, such as nitrilotrimethylenephosphonic acid, ethylenediaminetetra(methylenephosphonic acid), and hydroxyethylidenediphosphonic acid, or combinations of two or more of the above agents.

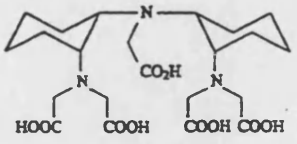
Preferred chelating residues contain polycarboxylic acid or carboxylate groups and include elements present in: ethylenediamine-N, N, N',N'-tetraacetic acid (EDTA); N,N, N',N'',N''-diethylenetriaminepentaacetic acid (DTPA); 1,4,7, 10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (DOTA); 1,4,7,10-tetraazacyclododecane-N,N',N''-triacetic acid (DO3A); 1-oxa-4,7,10-triazacyclododecane-N,N',N''-triacetic acid (OTTA); trans(1,2)-cyclohexanodiethylenetriamine pentaacetic acid (CDTPA);



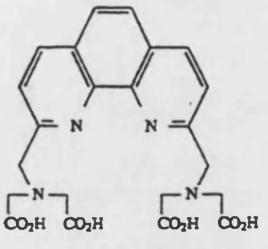
(B4A);



(P4A);



(DCDTPA); and



(PhcMT).

Such chelating compounds, including their preparation and manipulation are well known in the art. For example, the acid and anhydride forms of EDTA and DTPA are commercially available; methods for preparing B4A, P4A and TMT are described in U.S. Pat. No. 4,859,777; the disclosure of which is hereby incorporated by reference; and other suitable chelating groups are known in the art, and are described in PCT/US91/08253, and many other readily available references.

If the chelating residue is made of multiple chelating moieties or subunits, such subunits can be linked together by a linking group. Thus, more than one chelating moiety can be used to make up the chelating residue. If more than one chelating moiety is present in the chelating residue, these may be the same or different. Chelating moieties can be linked together using known chemistries. Thus the chelating residue can be one moiety or a "core" of chelating moieties. For example, a core of DTPA residues may be prepared by reacting DTPA dianhydride with a aliamine, such as ethylene diamine, to form a "core" of DTPA chelators. Other chelating residues, made up of multiple chelating moieties are well known in the art and are prepared by known chemistries as well.

For magnetic resonance imaging applications, $M^{(+n)}$ preferably represents a paramagnetic metal ion such as an ion of metals of atomic number 21 to 29, 42, 44 and 57 to 71, especially 57 to 71. Ions of the following metals are preferred: Cr, V, Mn, Fe, Co, Ni, Cu, Ce, Pr, Nd, Pro, Sin, Eu, Gd, Tb, Dy, Ho, Er, Tm and Yb. Especially preferred are Cr^{+3}, Cr^{+2}, V^{+2}, Mn^{+3}, Mn^{+2}, Fe^{+3}, Fe^{+2}, Co^{+2}, Gd^{+3} and Dy^{+3}. It is a particularly advantageous feature that polymers can be provided exhibiting a high substitution ratio, i.e., containing relatively large numbers of paramagnetic metal ions per molecule.

The cytotoxic agent can be a radioactive isotope, preferably a radioactive metal ion isotope. This radioactive metal isotope can be an ion of an isotope of a metal selected, for example, from Sc, Fe, Pb, Ga, Y, Bi, Mn, Cu, Cr, Zn, Ge, Mo, Tc, Rn, In, Sn, Re, Sr, Sin, Lu, Du, Sb, W,, Re, Po, Ta and Tl ions. In a prefered embodiment, radioisotopes which are also useful in diagnostic imaging applications are specifically contemplated. Thus this embodiment finds utility in imaging and therapy where either procedure can be performed in conjunction with or ancillary to the other. Preferred isotopes of radioactive metal ions for this embodiment include ^{44}Sc, $^{64,67}Cu$, ^{111}In, ^{212}Pb, ^{68}Ga, ^{90}Y, ^{153}Sm, ^{212}Bi, ^{99m}Tc and ^{188}Re for therapeutic and diagnostic imaging applications.

If a metal is chelated by the polymer, as for example, in imaging or therapy as described above, the metal content in the polymer can vary from about 0.1 up to about 20% based on the total weight of the polymer. For example in a magnetic resonance imaging embodiment, the polymer preferably contains a paramagnetic metal ion in an amount of from 1 to 25%, more preferably 2–20% by weight. In a therapeutic embodiment the radionuclide metal ion is present in roughly the same amounts as for imaging.

The PAG moiety in this composition can be capped at the terminus with a capping moiety selected from a hydrogen, hydroxy, alkyl, amine, or alkoxy. Preferred capping groups are hydrogen or hydroxyl groups. Thus capping is done by known chemistry, and precapped prepolymers are available. It is further contemplated that cyclic copolymers can be prepared.

The compositions of this invention can be prepared in water-soluble, water-dispersible or water-insoluble forms depending upon the intended application. The composition can have a molecular weight ranging from 10,000 to 1 million preferably 11,000 to 80,000. The preferred molecular weight varies according to the application as described below.

In addition to targeted delivery of the polymers of the invention, the polymer can be selectively delivered to specific cells, tissue types, or organs with or without the aid of a targeting agent. When no targeting agent is used such targeted delivery is based on size (hydrodynamic radius) and charge alone. The charge of the polymer can be altered by

judicious choice of the aminoacids used in the peptide component of the copolymer to suit the application. Of course, the size of the polymer can be chosen by altering the size of PAG or peptide used to prepare the polymer or by altering the degree of polymerization. The mechanism of the targeted delivery of polymer is thought to be based upon the passive biodistribution in tissues of the polymer. It is thought that this passive biodistribution can occur because the PAG component of the polymers allows free distribution of the polymers within the circulatory system, with low antigenicity or without interference by the mononuclear phagocytic system. Unlike hydrophobic polymers known in the art, which are taken up by the reticuloendothelial system, the polymers of the invention can be designed to be distributed to tissues without being metabolized. Thus the size and charge of the polymer in the tissue is a function of the size and charge of the polymer administered. Distribution of the unmetabolized polymer to tissues will be influenced by the nature of the local vascular endothelium in each tissue and the prescence or absence of a lymphatic system. Three general categories of vascular endothelium are sinusoidal epithelium, characterized by discontinuity and little or no basement membrane; fenestrated vascular endothelium; and continuous vascular endothelium, characterized by tight junctions and basement membrane. The lymphatic system is known to recirculate proteins and other molecules which can float freely in the plasma, but escape the circulatory system, exist for a time in tissue and then are returned to the circulatory system via the lymphatic system. The skilled artisan can determine which tissues will be passively targeted by the polymer by approximating the molecular weight or more preferably the hydrodynamic radius of known proteins diffusing through the tissue in a known given period.

Tissues such as bone marrow, liver and spleen tissue are characterized by sinusoidal endothelium, (which allows escape of large molecules from the circulating system into the surrounding tissue) thus larger polymer molecules are useful in passively targeting such tissues. Tissues such as found in the GI tract, kidney glomeruli, and endocrine gland tissue are characterized by fenestrated endothelium (which allows escape of smaller macromolecules from the circulatory system), thus slightly smaller polymer molecules are useful in passively targeting such tissues. Tissues such as muscle and lung tissue are characterized by continuous vascular endothelium (which allows small molecules to escape from the circulatory system into the surrounding tissue), thus smaller polymer molecules are useful in passively targeting these tissues.

For example, the hydrodynamic radius of albumin is approximately 37 Å, its molecular weight is 67 Kd, and its charge is known. It is known that the average half life for albumin circulation through tissue is approximately 24 hours, but this half life is longer in some tissues and shorter in others. Moreover, the concentration of albumin in certain tissues is appreciable and in other tissues albumin is nearly absent altogether. The skilled artisan can prepare a polymer of approximately the same size, or preferably the same hydrodynamic radius and charge, and expect a similar half life and concentration in tissues.

The skilled artisan will recognize that inflammation of tissues will perturb the normal physiology of that tissue and thus the half life and concentration of macromolecules, such as proteins or the polymer of the invention, in an inflamed tissue or inflamed tissue site. Thus the polymer finds utility in imaging and/or treating such inflamed tissues or inflamed tissue sites.

The skilled artisan will also appreciate that the absence of a lymphatic system in a tissue will perturb the concentration and increase the half life of macromolecules in a tissue because no convenient mechanism is provided for the scavenging of such macromolecules. Such is the case in growing tumors. One can deliver a cytotoxic agent, a pro-drug, or an imaging moiety to the growing tumor surface based on size of the polymer and on vasculature of the surrounding targeted tissue as described above. Thus dosing a cytotoxic agent will result in accumulation of such agent in the growing surface of the tumor.

Thus molecular weight and charge of the polymer may be tailored to the specific application based on tissue type, presence or absence of inflammation, tumor and/or vasculature type and presence or absence of a lymphatic system to provide a polymer with the correct characteristics for targeting the desired tissue.

The general synthetic methods for production of linear alternating polymers follow two related schemes (A and B) involving the reaction of a bis-(methylamino)-monomer with a bis(oxiranyl)-monomer described below. Compounds of the invention are prepared by chemical transformations which are conventional and known to those skilled in the art of chemistry. Furthermore, known transformations can be used for effecting changes in functional groups in the polymer or compounds used in preparing the polymer of the invention. For example, acylation of hydroxy- or amino-substituted species to prepare the corresponding esters or amides, respectively; simple aromatic and heterocyclic substitutions or displacements; cleavage of alkyl or benzyl ethers to produce the corresponding alcohols or phenols; and hydrolysis of esters or amides to produce the corresponding acids, alcohols or amines, preparation of anhydrides, acid halides, aldehydes, simple aromatic alkylation and the like as desired can be carried out.

Such transformations will provide suitable chelating agents and precursors thereof containing reactive functionality, including, for example, polycarboxylic acids in dianhydride form, di(sulfonyl chlorides), di(alkyl sulfates), di(vinyl sulfones), diesters, and the like. Such known transformations are also useful in attaching the chelator to the polymer or polymer precursor, and in preparing the polymer itself. However, as will be recognized by one skilled in the art, obtaining the desired product by some reactions will be better facilitated by blocking or rendering certain functional groups inert. This practice is well recognized in the art, see for example, Theodora Greene, *Protective Groups in Organic Synthesis* (1991). Thus when reaction conditions are such that they may cause undesired reactions with other parts of the molecule, for example in portions of the chelator intended to become ligands, the skilled artisan will appreciate the need to protect these reactive regions of the molecule and will act accordingly. For example, the chelating residue containing reactive functionality can be prevented from reacting to form undesired products by suitably blocking the chelating residue precursor which can be contacted with the reactive poly(alkylene oxide) moiety to form the polymer, and then the blocking group can be subsequently removed by techniques known in the art. For example, if hydroxy substituents are to be selectively present in the final polymer, they preferably should be temporarily blocked during polymerization, such as by formation of an alkyl ether from the hydroxyl by conventional blocking techniques to minimize formation of undesirable by products. However, by products which contain one or more linkages formed by unblocked reactive precursor groups in the backbone of the polymer are contemplated to be useful.

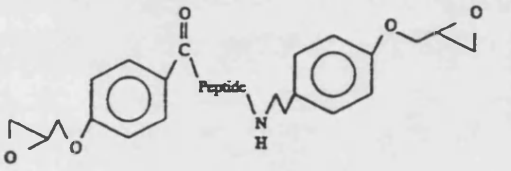
Small proteins or peptides may be incorporated into the polymer by methods as described hereinbelow. An advantage of this chemistry is that the N and C terminus of the peptide can be reversed or randomized in the polymer of the invention, reducing immunogenicity or masking peptide activity until the peptide is liberated.

Scheme A

Bis-(oxiranyl)-peptide Monomers (Apep) are Reacted with bis-(alkylamino)-PAG Derivative Monomers (Apag)

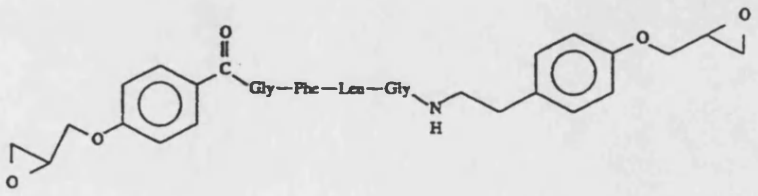
A linking group precursor is added to the PAG monomers at the terminal hydroxy. The reaction of the known linking group precursor with the known PAG moiety forms a (PAG)-linking group precursor radical. The precursor radical is chosen from aminoalkylamino, N-sarcosyl-aminoalkyl-amino, or N-sarcosylaminoalkylamino-N'-carboxy.

In this scheme, the peptide monomers, have 4-(oxiranylmethoxy) aryl radicals connected as linking group precursors using carboxy funtionality to attach to the N terminus of the peptide or amino functionality to attach to the C terminus of the peptide, thus forming amide bonds with the N terminus with the C terminus of the peptide monomer with the one end of each linking group precursor, and having an oxirane at the other end of each linking group precursor as shown by the example below:



This oxirane functionalized peptide is referred to as Apep.

As an example Apep can be;



and is combined with Bis(amino)PAG monomers (Apag), such as:

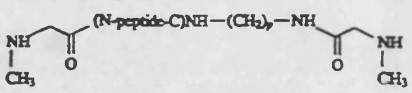
wherein R is lower alkyl.

Such PAG derivatives are prepared by known chemistry, for example; the preparation of an acid chloride from PAG monomers by $SOCl_2$, $COCl_2$ and the like, with subsequent reaction with a suitable diamine, or another suitable linking group, such as $—N(R)CH_2CONHCH_2CH_2NH_2$, or the like.

Scheme B

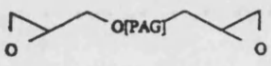
Alternatively, oxiranyl functionality can be used on PAG derivative monomers while using amino functionality on peptide derivative monomers. In this scheme, bis-(alkylamino)-peptide monomers (Bpep) are reacted with bis-(oxiranyl)-PAG monomers (Bpag). The peptide has a linking group precursor radical attached to the C and N termini so as to provide terminal amine functionality. Glycine or sarcosine can be used as the linking group precursor for the N terminus. The C terminus is attached to a $—NH(CH_2)_pNHCOCH_2NH(R)$ or $—NH(CH_2)_pNHCOCH_2NH_2$ linking group precursor radical which is derived from a diamine (wherein P is one to six, R is an alkyl radical, linear or branched, of 1 to about 4 carbons) and glycine or sarcosine. Thus the peptide is attached to the linking group precursor via amide linkages at both the N and C termini.

An example of Bpep is:



wherein p is 1 to about 6.

The bis(oxiranyl) PAG monomers (Bpag) of formula;



are known in the art. (See Y. Chen and M. Feng, Chinese Patent 86/104,089 (1987))

Thus it will be appreciated that the bis (alkylamine) and bis (oxiranyl) functionality may be on either the PAG moiety or the peptide moiety; so long as the polymerization takes place between a peptide and PAG, using the reaction of an amine and an epoxide.

Before, during or after polymerization, suitable chelating agents and precursors thereof may be attached to the polymer or polymer precursor. As described previously, a suitably blocked progenitor to the chelating agent or precursor thereof containing reactive functionality can be contacted with the reactive amino acid side chain incorporated into the polymer or polymer precursor to form the chelate-polymer or chelate polymer precursor, and then any blocking groups can be subsequently removed by techniques known in the art, thus avoiding formation of undesired by products.

The metallized polymer can be formed by contacting the unmetallized polymer sequentially or simultaneously with one or more sources of metal ions. This can be conveniently accomplished by adding one or more metal ion solutions or one or more metal ion solid salts or metal ion oxides, preferably sequentially, to a solution, preferably an aqueous solution, of the polymer. Thereafter, or between sequential addition of metal ions, the chelated polymer preferably is diafiltered in water to remove excess unbound metal.

The composition preferably is prepared in a water soluble, for example, an injectable form when used as magnetic resonance contrast agent for blood pool imaging, as a

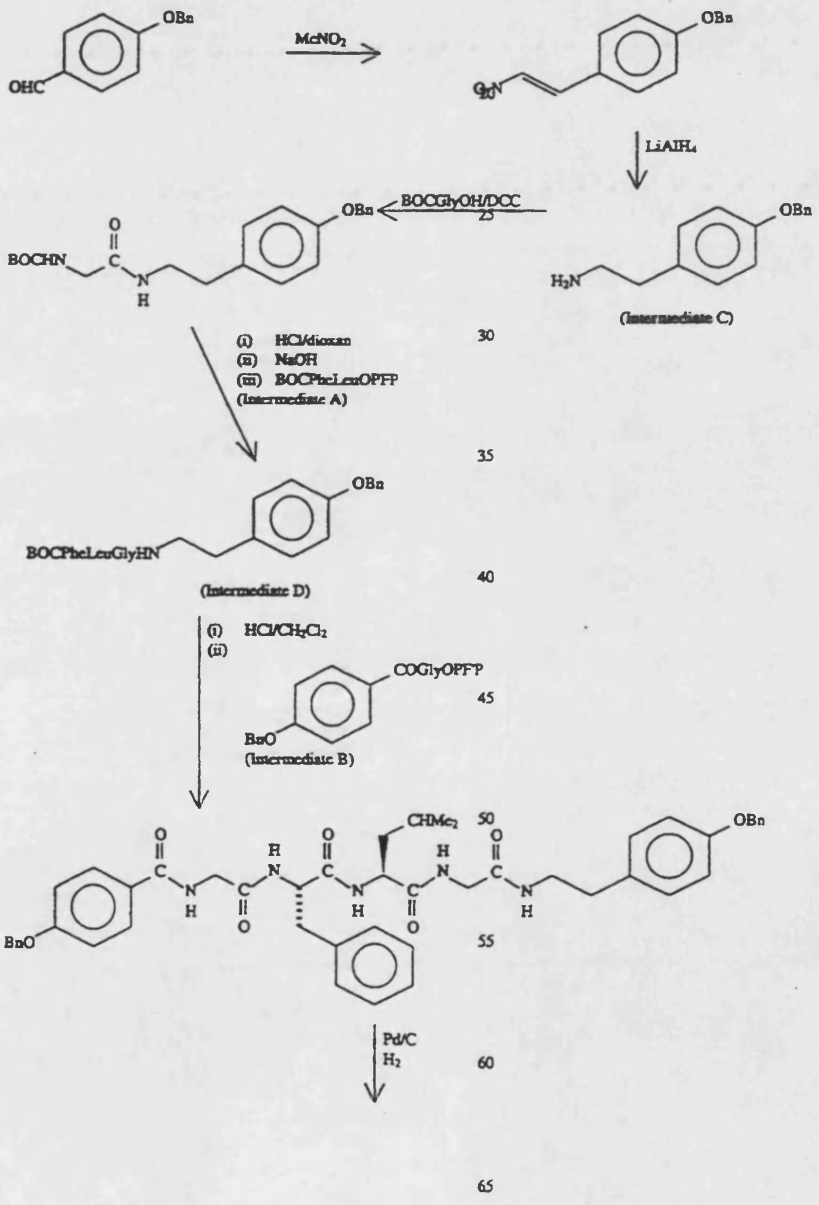
composition intended to be administered intravenously, and the like. The preparation of water-soluble compositions of molecular weight 10,000 to 50,000 can be accomplished by known methods by one skilled in the art.

The following example illustrates the preparation of an example of a compound of formula A. It is understood that the example presented does not limit the claims or invention claimed.

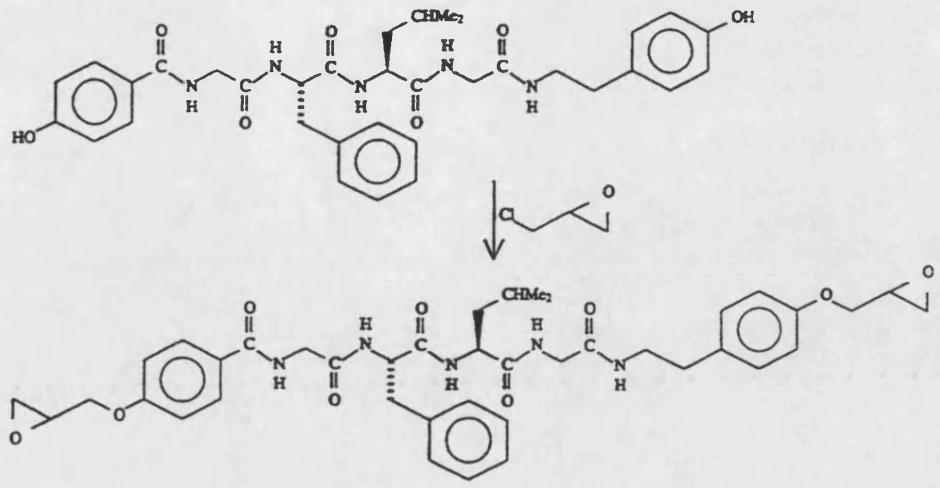
Example A

The synthesis of the compound prepared by Method A, giving a compound of formula A is achieved according to the following scheme;

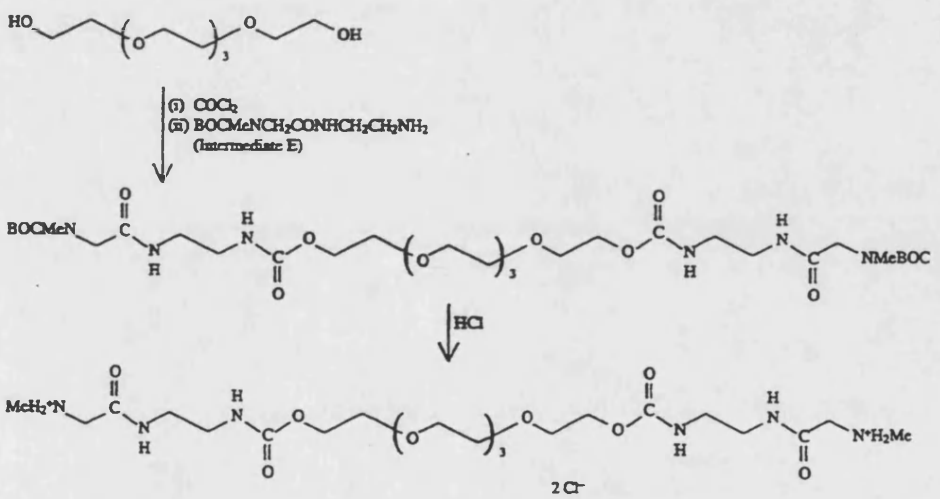
Preparation of the Peptide Portion of Example A



Preparation of the Peptide Portion of Example A (Cont.)



Preparation of PAG Portion of Example A



EXAMPLE A

Intermediate A

1. N-(N-(1,1-Dimethylethoxycarbonyl)phenylalanyl)leucine pentafluorophenylester. N-(N-(1,1-Dimethylethoxycarbonyl)-phenylalanyl)leucine (23.0 g, 61 mmol) (prepared by a literature method[Anderson, G. W.; McGregor, A. C., t-Butoxycarbonyl amino acids and their use in peptide synthesis, J. Am. Chem. Soc., 1957, 79, 6180–6183]) was stirred with pentafluorophenol (11.2 g, 61 mmol) and dicyclohexylcarbodiimide (12.5 g, 61 mmol) in tetrahydrofuran (170 mL) for 1 h at 0° C. The suspension was filtered. The solvent was evaporated from the filtrate under reduced pressure. The residue, in dichloromethane, was washed twice with saturated aqueous sodium hydrogen carbonate and with water. The solution was dried with anhydrous magnesium sulphate and the solvent was evaporated under reduced pressure to give N-(N-(1,1-dimethylethoxycarbonyl)phenylalanyl)leucine pentafluorophenyl ester (28.0 g, 85%).

Intermediate B

1. 4-(Phenylmethoxy)benzoic acid. In a modification of the literature method [E. L. Elied, R. P. Anderson, Reactions of esters with targeting amines. I. Benzyl esters from methyl esters and benzyldimethylamine, *J. Am. Chem. Soc.*, 1952, 74, 547–549] a mixture of 4-hydroxybenzoic acid (27.6 g, 200 mmol), chloromethylbenzene (57.0 g, 450 mmol), potassium carbonate (50 g) and sodium iodide (25 g) was boiled under reflux in acetonitrile (500 mL) for 16 h. The suspension was filtered and the solvent was evaporated from the filtrate under reduced pressure. The residue was recrystallised from ethanol to give phenylmethyl 4-(phenylmethoxy)benzoate (48.8 g, 76%). Phenylmethyl 4-(phenylmethoxy)benzoate (48.8 g, 150 mmol) was boiled under reflux with aqueous sodium hydroxide (2M; 250 mL) and ethanol (250 mL) for 4 h. The ethanol was evaporated under reduced pressure. Water (1000 mL) was added. The white solid was collected by filtration, warmed to 65° C. with aqueous sulphuric acid (2M; 300 mL) for 1 h and extracted

with warm ethyl acetate. The ethyl acetate solution was dried with anhydrous magnesium sulphate and the solvent was evaporated under reduced pressure to give 4-(phenylmethoxy)benzoic acid (27.15 g, 80%). The filtrate was washed twice with diethyl ether, acidified by addition of sulphuric acid (2M) and extracted with diethyl ether. Evaporation of the diethyl ether gave a further portion of 4-(phenylmethoxy)benzoic acid (6.0 g, 18%). The total yield was 98%.

2. 4-(Phenylmethoxy)benzoyl chloride. 4-(Phenylmethoxy)benzoic acid (500 mg, 2.2 mmol) was stirred with oxalyl chloride (280 mg, 2.2 mmol) and dimethylformamide (25 mg) in 1,4-dioxan (25 mL) for 20 min. The solvent and catalyst were evaporated under reduced pressure. The residue was recrystallised from hexanes to give 4-(phenylmethoxy)benzoyl chloride (460 mg, 85%).

3. N-(4-(Phenylmethoxy)benzoyl)glycine methyl ester. 4-(Phenylmethoxy)benzoyl chloride (13.64 g, 55.5 mmol) in dichloromethane (90 mL) was added dropwise to glycine methyl ester hydrochloride (7.66 g, 61 mmol) and triethylamine (11.78 g, 116.5 mmol) in dichloromethane (250 mL). The mixture was stirred for 16 h. The suspension was filtered. The solvent was evaporated from the filtrate under reduced pressure. The residue was recrystallised from dichloromethane/hexane to give N-(4-(phenylmethoxy)benzoyl)glycine methyl ester (14.75 g, 89%).

4. N-(4-(Phenylmethoxy)benzoyl)glycine pentafluorophenyl ester. N-(4-(Phenyl-methoxy)benzoyl)glycine methyl ester (14.75 g, 49.2 mmol) was boiled under reflux with methanolic sodium hydroxide (1M) (80 mL) for 2 h. The solvent was evaporated under reduced pressure. The residue was dissolved in water and was acidified by addition of aqueous hydrochloric acid. The suspension was extracted with ethyl acetate. The extract was washed with saturated brine and was dried with anhydrous magnesium sulphate. The solvent was evaporated under reduced pressure to give N-(4-(phenylmethoxy)benzoyl)glycine (6.59 g, 47%). Dicyclohexylcarbodiimide (720 mg, 3.5 mmol) was added to N-(4-(phenylmethoxy)benzoyl)glycine (100 g, 3.5 mmol) in dry tetrahydrofuran (100 mL) and the mixture was taken to 0° C. Pentafluorophenol (640 g, 3.5 mmol) was added dropwise and the mixture was stirred for 17 h at 0° C. The suspension was filtered and the solvent was evaporated from the filtrate under reduced pressure. The residue was dissolved in ethyl acetate (200 mL) and was washed with saturated aqueous sodium hydrogen carbonate (2×75 mL), with aqueous sulphuric acid (10%) and with water. The solution was dried with anhydrous magnesium sulphate and the solvent was evaporated under reduced pressure to give N-(4-(phenylmethoxy)benzoyl)glycine pentafluorophenyl ester (Intermediate B) (1.5 g, 95%).

Intermediate C

1. 1-(2-Nitroethenyl)-4-(phenylmethoxy)benzene. In a modification of the literature method [M. Hoequanx, B. Macot, G. Reclenith, C. Viel, M. Brunaub, J. Nauamo, C. Lacoun and C. Cozeubon, Diazoestrones and analogs. I. Pharmacological study and syntheses of heterosteroid analogs to establish structure—analgesic activity relationships, *Eur. J. Med. Chem.*, 1983, 19, 319–329], to 4-(phenylmethoxy)benzaldehyde (28 g, 132 mmol) in ethanol (900 mL) at 5° C. was added nitromethane (16.1 g, 264 mmol). Sodium hydroxide (13.2 g, 330 mmol) in ethanol (200 mL) was added dropwise and the mixture was stirred for 30 min at 5° C. The mixture was poured into a mixture of hydrochloric acid (9M; 136 mL) and water (208 mL). The precipitate was collected by filtration and was recrystallised from ethanol to give 1-(2-nitroethenyl)-4-(phenylmethoxy)benzene (14.0 g, 42%).

2. 2-(4-(Phenylmethoxy)phenyl)ethylamine. Lithium aluminum hydride (8.48 g, 223 mmol) was suspended in dry diethyl ether (600 mL). 1-(2-Nitroethenyl)-4(phenylmethoxy)benzene (13.9 g, 55 mmol) was extracted into this mixture using a Soxhlet apparatus. The mixture was boiled under reflux for 16 h. Water (7.38 mL) was added, followed by aqueous sodium hydroxide (20%; 5.53 mL) and water (27.8 mL). The suspension was filtered. The solvent was evaporated from the filtrate under reduced pressure to give 2-(4-(phenylmethoxy)phenyl)ethylamine (11.25 g, 91%).

Intermediate D

1. N-(1,1-Dimethylethoxycarbonyl)glycine N-(2-(4-phenylmethoxy)phenyl)ethyl)amide. N-(1,1-Dimethylethoxycarbonyl)glycine (850 mg, 4.85 mmol) was stirred with dicyclohexylcarbodiimide (1.00 g, 4.85 mmol) and 2-(4-(phenylmethoxy)phenyl)ethylamine (Intermediate C) (1.00 g, 4.4 mmol) in dry tetrahydrofuran (30 mL) for 16 h. The suspension was filtered and the solvent was evaporated from the filtrate under reduced pressure. The residue was dissolved in ethyl acetate and was washed with aqueous sulphuric acid (10%) and with saturated brine. The solution was dried with anhydrous magnesium sulphate and the solvent was evaporated under reduced pressure to give N-(1,1dimethylethoxycarbonyl)glycine N-(2-(-(phenylmethoxy)phenyl)ethyl)amide (1.65 g, 98%).

2. Glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide. N-(1,1-Dimethylethoxycarbonyl)glycine N-(2-(4-(phenyl-)ethyl)amide (2.01 g, 5.23 mmol) was treated with excess hydrogen chloride in 1,4-dioxan (45 mL) for 2 h. The solid was collected by filtration and was dissolved in water and ethyl acetate. Aqueous sodium hydroxide was added to basify the solution to pH 9. The ethyl acetate solution was dried with anhydrous magnesium sulphate and the solvent was evaporated under reduce pressure to give glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide (1.15 g, 77%).

3. N-(N-(N-(1,1-Dimethylethoxycarbonyl)phenylalanyl)leucyl)glycine N-(2-(4-(phenylmethoxy)phenyl-)ethyl)amide. N-(N-(1,1-Dimethylethoxycarbonyl)phenylalanyl)leucine pentafluorophenyl ester (1.19 g, 2.18 mmol) (Example A, Intermediate A) in tetrahydrofuran (30 mL) was added dropwise to glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide (620 mg, 2.18 mmol), N,N-diisopropylethylamine (310 mg, 2.4 mmol) and 1-hydroxybenzotriazole (20 mg) in tetrahydrofuran (30 mL) and the mixture was stirred for 16 h. The solvent was evaporated under reduced pressure. The residue, in ethyl acetate, was washed with aqueous sulphuric acid (10%) and with saturated aqueous sodium hydrogen carbonate. The solution was dried with anhydrous magnesium sulphate and the solvent was evaporated under reduced pressure. The residue was triturated with diethyl ether and the solid was collected by filtration to give N-(N-(N-(1,1-dimethylethoxycarbonyl)phenylalanyl)leucyl)glycine N-(2-(4-(phenylmethoxy)phenyl)-ethyl)amide (360 mg, 26%) (Intermediate D).

Intermediate E

1. N-(1,1-Dimethylethoxycarbonyl)sarcosine 2,4,5-trichlorophenyl ester. N-(1,1-Dimethylethoxycarbonyl)sarcosine (10.0 g, 53 mmol) was stirred with 2,4,5-trichlorophenol (10.6 g, 53 mmol) and dicyclohexylcarbodiimide (10.9 g, 53 mmol) in ethyl acetate (100 mL) at −10° C. for 2.5 h. The suspension was filtered and the solvent was evaporated under reduced pressure from the filtrate. The residue was dissolved in ethyl acetate. The suspension was filtered and the solvent was evaporated under reduced pressure from the filtrate to give N-(1,1-dimethylethoxycarbonyl)sarcosine 2,4,5-trichlorophenyl ester (19.3 g, 98%).

2. N-(1,1-Dimethylethoxycarbonyl)sarcosine N-(2-aminoethyl)amide. N-(1,1-Dimethylethoxycarbonyl)sarcosine 2,4,5-trichlorophenyl ester (12.7 g, 34.5 mmol) in dichloromethane (50 mL) was added during 30 min to ethane-1, 2-diamine (20.7 g, 345 mmol) in dichloromethane (150 mL) and the solution was stirred for a further 2 h. The solution was washed with water and with 10% aqueous sodium carbonate and was dried with anhydrous magnesium sulphate. The solvent was evaporated under reduced pressure to give N-(1,1-dimethylethoxycarbonyl)sarcosine N-(2-aminoethyl)amide (6.9 g, 86%).

3. Bis(2-(2-(N-(2-N-(1,1-Dimethylethoxycarbonyl)sarcosyl)aminoethyl)aminocarboxy)ethoxy)ethoxy)ethane. Bis(2-hydroxyethoxy)ethoxy)ethane (5.0 g, 21 mmol) was boiled in toluene (120 mL) for 20 h with azeotropic removal of water. The resulting solution was cooled to 20° C. Dichloromethane (35 mL) was added, followed by phosgene (1.93M in dichloromethane, 109 mL, 210 mmol). The solution was stirred for 4 h. The solvent and excess reagent were evaporated under reduced pressure from a portion (30 mL) of this solution to give crude bis(2-(2-(chlorocarboxy-)ethoxy)ethoxy)ethane (900 mg, 2.5 mmol). This material was dissolved in dichloromethane (50 mL). To this solution was added triethylamine (1.26 g, 12.5 mmol) and 4-(dimethylamino)pyridine (20 mg). N-(1,1-dimethylethoxycarbonyl)sarcosine N-(2-aminoethyl)amide (1.73 g, 7.5 mmol) (Intermediate E2)in dichloromethane (100 mL) was then added dropwise during 40 min. The solution was stirred for 20 h before being washed with water, 10% aqueous sulphuric acid and water. The solution was dried with anhydrous magnesium sulphate and the solvent was evaporated under reduced pressure to give bis(2-(2-(N-(2-(N-(1,1-dimethylethoxycarbonyl)sarcosyl)amino-ethyl)aminocarboxy)ethoxy)ethoxy)ethane (1.1 g, 59%).

4. Bis(2-(2-(N-(2-sarcosylaminoethyl)aminocarboxy)ethoxy)ethoxy)ethane dihydrochloride. Bis(2-(2-(N-(2-(N-(1,1-dimethylethoxycarbonyl)sarcosyl)aminoethyl)aminocarboxy)ethoxy)ethoxy)ethane (752 mg, 1 mmol) was treated with excess hydrogen chloride in dichloromethane for 2 h. Evaporation of the solvent gave bis(2-(2-(N-(2-sarcosylaminoethyl)aminocarboxy)ethoxy)ethoxy)ethane dihydro-chloride (550 mg, quantitative).

Preparation of the Peptide Portion of Example A

1. N-(N-Phenylalanylleucyl)glycine N-(2-(4-(phenylmethoxy)phenyl) ethyl)amide hydrochloride. N-(N-(1,1-Dimethylethoxycarbonyl)phenylalanylleucyl) glycine N-(2-(4-(phenylmethoxy) phenyl)ethyl)amide (3.89 g, 6.05 mmol) was treated with excess hydrogen chloride in dichloromethane (200 mL) for 3 h. The solvent and excess reagent were evaporated under reduced pressure. The residual oil was triturated with diethyl ether to give N-(N-phenylalanylleucyl)glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide hydrochloride (3.26 g, 93%).

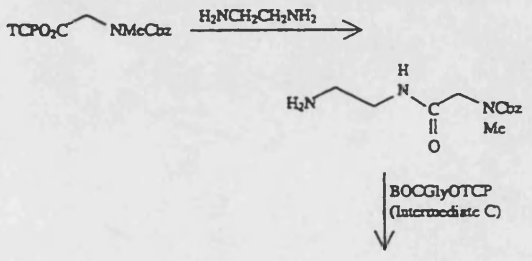
2. N-(N-(N-(N-(4-(Phenylmethoxy)benzoyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide. N-(N-Phenylalanylleucyl)g lycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide hydrochloride (165 mg, 284 mol) was stirred with N,N-diisopropylethylamine (100 mg, 774 mol), 4-(dimethylamino)pyridine (10 mg) and 1-hydroxybenzotriazole (10 mg) in dry dichloromethane (5 mL) until all solid dissolved. N-(4-(Phenylmethoxy)benzoyl) glycine pentafluorophenyl ester (117 mg, 258 mol) (Example A, Intermediate B)in chloroform (10 mL) was added dropwise during 30 min and the reaction mixture was stirred for 5 h. The solvent was evaporated under reduced pressure. Column chromatography (silica gel; chloroform/methanol 50:1) of the residue gave N-(N-(N-(N-(4-(phenylmethoxy)benzoyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide (170 mg, 81%).

3. N-(N-(N-(N-(4-Hydroxybenzoyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(4-hydroxyphenyl)ethyl)amide. N-(N-(N-(N-(4-(Phenylmethoxy)benzoyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(4-(phenylmethoxy)phenyl)ethyl)amide (444 mg, 547 mol) in ethanol (45 mL) was stirred vigorously with palladium on charcoal (10%; 50 mg) and hydrogen for 12 h. The suspension was filtered through diatomaceous earth. The solvent was evaporated from the filtrate under reduced pressure to give N-(N-(N-(N-(4-hydroxybenzoyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(4-hydroxyphenyl)ethyl)amide (304 mg, 88%).

4. N-(N-(N-(N-(4-(Oxiranylmethoxy)benzoyl)glycyl) phenylalanyl)leucyl) glycine N-(2-(4-(oxiranylmethoxy)phenyl)ethyl)amide. N-(N-(N-(N-(4-Hydroxybenzoyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(4-hydroxyphenyl) ethyl)amide (106 mg, 0.131 mol) was suspended in water (12 mL) containing sodium hydroxide (52.3 mg, 1.31 mmol). Chloromethyloxirane (604 mg, 6.5 mmol) in methanol (10 mL) was added, followed by phenylmethyltrimethylammonium hydroxide (40% aqueous solution, 90 mg). The solution was stirred for 48 h at 40° C. The solvent and excess reagent were evaporated under reduced pressure. The residue was dissolved in ethyl acetate and was washed with water. The solution was dried with anhydrous magnesium sulphate. The solvent was evaporated under reduced pressure. Column chromatography (silica gel; ethyl acetate, then ethyl acetate/methanol 39:1, then ethyl acetate/methanol 19:1, then ethyl acetate/methanol 9:1) gave N-(N-(N-(N-(4-(oxiranylmethoxy)benzoyl)glycyl) phenylalanyl)leucyl)glycine N-(2-(4-(oxiranylmethoxy)phenyl)ethyl)amide (26.5 mg, 27%).

5. Polymer A. It is contemplated that N-(N-(N-(N-(4-(Oxiranylmethoxy)benzoyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(4-(oxiranylmethoxy)phenyl)ethyl)amide is boiled under reflux with anhydrous sodium carbonate and bis(2-(2-(N-(2-sarcosylaminoethyl)aminocarboxy)ethoxy)ethoxy)ethane——dihydrochloride (Intermediate E) in ethanol for 6 h, giving the polymer of formula A.

Preparation of Peptide Portion of Example B



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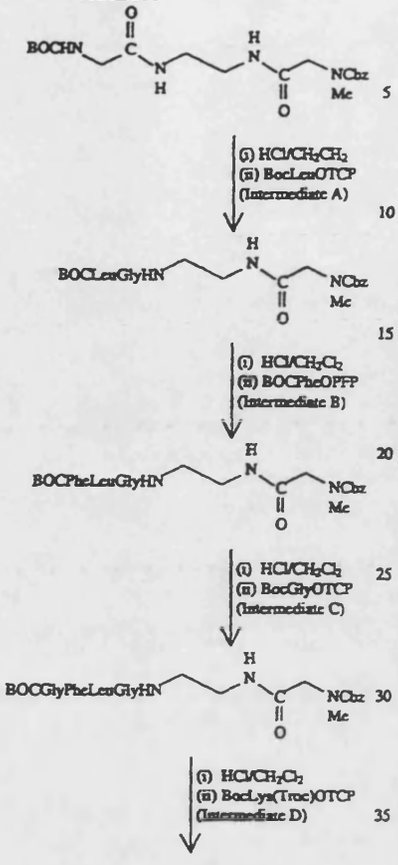
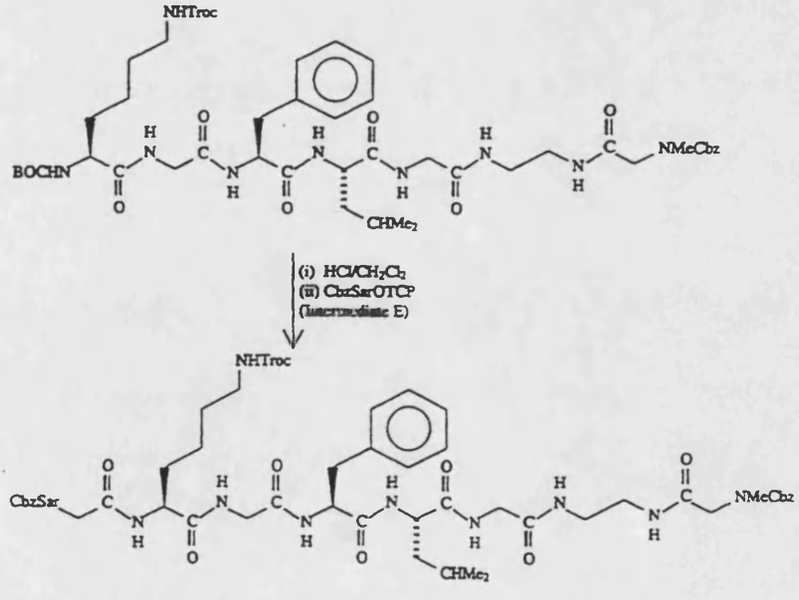


Preparation of Peptide Portion of Example B



EXAMPLE B

PREPARATION OF INTERMEDIATES

Intermediate A

N-(1,1-Dimethylethoxycarbonyl)leucine 2,4,5-trichlorophenyl ester. N-(1,1-Dimethylethoxycarbonyl)leucine (6.32 g, 15 mmol) was stirred with 2,4,5-trichlorophenol (3.01 g, 15.2 mmol) and dicyclohexylcarbodiimide (3.14 g, 15.2 mmol) in ethyl acetate (50 mL) at −10° C. for 4 h. The suspension was filtered and the solvent was evaporated under reduced pressure from the filtrate. The residue was dissolved in ethyl acetate. The suspension was filtered and the solvent was evaporated under reduced pressure from the filtrate to give N-(1,1-dimethylethoxycarbonyl)leucine 2,4,5-trichlorophenyl ester (6.2 g, 99%).

Intermediate B

N-(1,1-Dimethylethoxycarbonyl)phenylalanine pentafluorophenyl ester. N-(1,1-Dimethylethoxycarbonyl)phenylalanine (6.36 g, 24 mmol) in ethyl acetate (50 mL) at 0° C. was added to dicyclohexylcarbodiimide (4.95 g, 24 mmol) and pentafluorophenol (4.42 g, 24 mmol) in ethyl acetate (50 mL) at 0° C. The mixture was stirred for 2.75 h at 0° C. The suspension was filtered and the solvent was evaporated under reduced pressure from the filtrate. The residue was dissolved in ethyl acetate. The suspension was filtered and the solvent was evaporated under reduced pressure from the filtrate to give N-(1,1-dimethylethoxycarbonyl)phenylalanine pentafluorophenyl ester (10.32 g, quantitative).

Intermediate C

N-(1,1-Dimethylethoxycarbonyl)glycine 2,4,5-trichlorophenyl ester. N-(1,1-Dimethylethoxycarbonyl)glycine (6.12 g, 35 mmol) was stirred with 2,4,5-trichlorophenol (6.91 g, 35 mmol) and dicyclohexylcarbodiimide (7.22 g, 35 mmol) in ethyl acetate (100 mL) at 0° C. for 4 h. The suspension was filtered and the solvent was evaporated under reduced pressure from the filtrate. The residue was

dissolved in ethyl acetate. The suspension was filtered and the solvent was evaporated under reduced pressure from the filtrate to give N-(1,1-dimethylethoxycarbonyl)glycine 2,4,5-trichlorophenyl ester (12.4 g, quantitative).

Intermediate D

1. N^{α}-(1,1-Dimethylethoxycarbonyl)-N^{ϵ}-(2,2,2-trichloroethoxycarbonyl)lysine. In a modification of a literature method [Yajima, H.; Watanabe, H.; Okamoto, M., Studies on peptides. XXXIII. N^{ϵ}-β,β,β-Trichloroethyloxycarbonyllysine, *Chem. Pharm. Bull*, 1971, 19, 2185–2189], lysine monohydrochloride (9.14 g, 50 mmol) was stirred under reflux with copper (II) carbonate (21.6 g, 75 mmol) in water (180 mL) for 3 h. The solution was filtered while hot and the filtrate was cooled to 20° C. 2,2,2-Trichloroethyl chloroformate (15.9 g, 75 mmol) and aqueous sodium carbonate (13.3 g, 125 mmol in 40 mL) were added alternately in portions to the filtrate during 30 min and the mixture was stirred vigorously at 0° C. for 20 h. The blue precipitate was collected and was boiled under reflux with ethylenediaminetetraacetic acid disodium salt (18.6 g, 100 mmol) in water (200 mL) for 2 h. The solution was cooled to 0° C. for 20 hrs and crude N^{ϵ}-(2,2,2-trichloroethoxycarbonyl)lysine was collected as a gummy solid. This material was dissolved in water (75 mL) and triethylamine (20.2 g, 200 mmol) was added, followed by di-t-butyl dicarbonate (13.64 g, 62 mmol) and 1,4-dioxan (30 mL). The mixture was stirred vigorously for 3 d. The mixture was washed with diethyl ether. Ethyl acetate was added to the aqueous phase and the mixture was acidified by careful addition of cold 10% aqueous sulphuric acid. The ethyl acetate phase was washed with water and dried with anhydrous magnesium sulphate. The solvent was evaporated under reduced pressure to give N^{α}-(1,1-dimethylethoxycarbonyl)-N^{ϵ}-(2,2,2-trichloroethoxycarbonyl)lysine (12.32 g, 55%).

2. N^{α}-(1,1-Dimethylethoxycarbonyl)-N^{ϵ}-(2,2,2-trichloroethoxycarbonyl)lysine 2,4,5-trichlorophenyl ester. N^{α}-(1,1-Dimethylethoxycarbonyl)-N^{ϵ}-(2,2,2-trichloroethoxy-carbonyl)lysine (6.32 g, 15 mmol) was stirred with 2,4,5-trichlorophenol (2.96 g, 15 mmol) and dicyclohexylcarbodiimide (3.10 g, 15 mmol) in ethyl acetate (100 mL) at 0° C. for 20 h. The suspension was filtered and the solvent was evaporated under reduced pressure from the filtrate. The residue was dissolved in ethyl acetate. The suspension was filtered and the solvent was evaporated under reduced pressure from the filtrate to give N^{α}-(1,1-dimethylethoxycarbonyl)-N^{ϵ}-(2,2,2-trichloroethoxy-carbonyl)lysine 2,4,5-trichlorophenylester (7.50 g, 97%).

Intermediate E

1. N-(Phenylmethoxycarbonyl)sarcosine 2,4,5-trichlorophenylester. N-(Phenylmethoxycarbonyl)sarcosine (4.0 g, 18 mmol) was stirred with 2,4,5-trichlorophenol (3.53 g, 18 mmol) and dicyclohexylcarbodiimide (3.69 g, 18 mmol) in ethyl acetate (40 mL) at −10° C. for 1 h, then at 20° C. for 20 h. The suspension was cooled to 0° C. The suspension was filtered and the solvent was evaporated under reduced pressure from the filtrate. The residue was dissolved in ethyl acetate. The suspension was filtered and the solvent was evaporated under reduced pressure from the filtrate to give N-(phenylmethoxycarbonyl)sarcosine 2,4,5-trichlorophenyl ester (7.2 g, quantitative).

2. N-(Phenymethoxycarbonyl)sarcosine pentafluorophenyl ester. N-(Phenylmethoxycarbonyl)sarcosine (3.0 g, 13.4 mmol) was stirred with pentafluorophenol (2.46 g, 13.4 mmol) and dicyclohexylcarbodiimide (2.32 g, 13.4 mmol) in ethyl acetate (30 mL) at 0° C. for 2 h. The suspension was filtered and the solvent was evaporated under reduced pressure from the filtrate. The residue was dissolved in ethyl acetate. The suspension was filtered and the solvent was evaporated under reduced pressure from the filtrate to give N-(phenylmethoxycarbonyl)sarcosine pentafluorophenyl ester (4.66 g, 89%).

Intermediate F

1. 5-(4-Nitrophenyl)-10,15,20-triphenyl-21H,23H-porphine. Fuming nitric acid (density 1.5 mL^{-1}) (2.26 mL) was added during 2 h to 5,10,15,20-tetraphenyl-21H,23H-porphine (2.00 g, 3.26 mmol) in chloroform (ethanol-free) (300 mL). The mixture was washed with water (5×300 mL) and was dried with anhydrous sodium carbonate and anhydrous magnesium sulphate. The solvent was evaporated under reduced pressure. Chromatography (silica gel; dichloromethane/hexane 2:1) of the residue gave 5-(4-nitrophenyl)-10,15,20-triphenyl-21H,23H-porphine (1.17 g, 55%).

2. 4-(10,15,20-Triphenyl-21H,23H-porphin-5-yl)benzeneamine. Tin(II) chloride dihydrate (595 mg, 2.6 mmol) was added to 5-(4-nitrophenyl)-10,15,20-triphenyl-21H,23H-porphine (580 mg, 0.88 mmol) in aqueous hydrochloric acid (9M, 20 mL) and the mixture was stirred at 65° C. for 2 h. The solution was allowed to cool and was added to water (70 mL). Concentrated aqueous ammonia was added until the solution was basified to pH 8. The suspension was extracted with chloroform (9×75 mL). The chloroform fractions were combined and were dried with anhydrous magnesium sulphate. The solvent was evaporated under reduced pressure. Chromatography (silica gel; dichloromethane/hexane 5:1) of the residue gave 4-(10,15,20-triphenyl-21H,23H-porphin-5-yl)benzeneamine (462 mg, 84%).

3. 4-Oxo-4-(4-(10,15,20-triphenyl-21H,23H-porphin-5-yl)phenylamino)butanoic acid. 4-(10,15,20-Triphenyl-21H,23H-porphin-5-yl)benzeneamine (450 mg, 0.72 mmol) was dissolved in chloroform (ethanol-free) (10 mL) with warming. Succinic anhydride (tetrahydrofuran-2,5-dione) (64 mg, 0.72 mmol) was added and the mixture was boiled under reflux for 2.5 h. A further portion of succinic anhydride (32 mg, 0.36 mmol) was added and boiling under reflux continued for a further 2 h. The mixture was cooled to ambient temperature for 16 h. The precipitated solid was collected by filtration to give 4-oxo-4-(4-(10,15,20-triphenyl-21H,23H-porphin-5-yl)phenylamino)butanoic acid (460 mg, 89%).

Preparation of Peptide Portion of Example B

1. N-(Phenylmethoxycarbonyl)sarcosine N-(2-aminoethyl)amide. N-(Phenylmethoxycarbonyl)sarcosine pentafluorophenyl ester (3.5 g, 9.2 mmol) in dichloromethane (40 mL) was added during 30 min to ethane-1,2-diamine (10.8 g, 180 mmol) in dichloromethane (300 mL) and the solution was stirred for a further 2 h. The solution was washed with water and with 10% aqueous sodium carbonate and was dried with anhydrous magnesium sulphate. The solvent was evaporated under reduced pressure to give N-(phenylmethoxycarbonyl)sarcosine N-(2-aminoethyl)amide (2.1 g, 88%). This material was also prepared similarly from N-(phenylmethoxycarbonyl)sarcosine 2,4,5-trichlorophenyl ester.

2. N-(1,1-Dimethylethoxycarbonyl)glycine N-(2-(N-phenylmethoxycarbonyl)sarcosylamino)ethyl)amide. N-(Phenylmethoxycarbonyl)sarcosine N-(2-aminoethyl)amide (3.71 g, 14 mmol) was stirred with N-(1,1-dimethylethoxycarbonyl)glycine 2,4,5-trichlorophenyl ester (4.96 g, 14 mmol, Example B, Intermediate C) and N,N-diisopropylethylamine (1.99 g, 15.4 mmol) in dichloromethane (100 mL) for 20 h. The solution was washed with cold 10% aqueous sulphuric acid (2×) and with saturated aqueous sodium hydrogen carbonate and was dried with anhydrous

magnesium sulphate. The solvent was evaporated under reduced pressure. Chromatography (silica gel; ethyl acetate/methanol 10:1, then ethyl acetate/methanol 5:1, then ethyl acetate/methanol 3:1) of the residue gave N-(1,1-dimethylethoxycarbonyl)glycine N-(2-(N-phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (2.12 g, 37%).

3. Glycine N-(2-(N-phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride. N-(1,1-Dimethylethoxycarbonyl)glycine N-(2-(N-phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (2.04 g, 4.95 mmol) was treated with excess hydrogen chloride in dichloromethane (50 mL) for 1 h. The solvent and excess reagent were evaporated under reduced pressure to give glycine N-(2-(N-phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (1.5 g, quantitative).

4. N-(N-(1,1-Dimethylethoxycarbonyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide. N-(1,1-Dimethylethoxycarbonyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (5.22 g, 8 mmol) was treated with excess hydrogen chloride in dichloromethane (50 mL) for 1 h. Water (50 mL) was added and the mixture was stirred vigorously for 15 min. The solvent and excess reagent were evaporated from the aqueous layer under reduced pressure to give crude glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride as a white solid. This material was stirred with N,N-diisopropylethylamine (3.231 g, 25 mmol) and N-(1,1-dimethylethoxycarbonyl)leucine 2,4,5-trichlorophenyl ester (3.19 g, 7.8 mmol) (Example II, Intermediate A) in dimethylformamide (30 mL) for 3 d. The solvent was evaporated under reduced pressure. The residue was dissolved in ethyl acetate and was washed with aqueous sodium hydroxide (5%), aqueous sulphuric acid (10%) and water and was dried with anhydrous magnesium sulphate. Evaporation of the solvent under reduced pressure gave N-(N-(1,1-dimethylethoxycarbonyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (3.26 g, 78%).

5. N-Leucylglycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride. N-(N-(1,1-Dimethylethoxycarbonyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (3.26 g, 6.1 mmol) was treated with excess hydrogen chloride in dichloromethane (40 mL) for 1 h. The solvent and excess reagent were evaporated under reduced pressure to give N-leucylglycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (2.65 g, quantitative).

6. N-(N-(N-(1,1-Dimethylethoxycarbonyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl) amide. N-(1,1-Dimethylethoxycarbonyl)phenylalanine pentafluorophenyl ester (2.65 g, 6.1 mmol) (Example B, Intermediate B) was added to N-leucylglycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (2.81 g, 6.16 mmol), N-N-diisopropylethylamine (1.75 g, 13.5 mmol) and 4-(dimethylamino)pyridine (10 mg) in dichloromethane (30 mL) and the mixture was stirred for 2 d. The solution was then washed with cold aqueous sulphuric acid (10%), aqueous sodium carbonate (10%) and saturated brine. The solution was dried with anhydrous magnesium sulphate and the solvent was evaporated under reduced pressure. Chromatography (silica gel; chloroform/methanol 1:1) gave N-(N-(N-(1,1-dimethylethoxycarbonyl)phenylalanyl)leucyl)glycine N-(2-(N(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (1.94 g, 46%).

7. N-(N-Phenylalanylleucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethylamyidehydrochloride. N-(N-(N-(1,1-Dimethylethoxycarbonyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (1.94 g, 2.8 mmol) was treated with excess hydrogen chloride in dichloromethane (25 mL) for 1 h. The solvent and excess reagent were evaporated under reduced pressure. The residue was dissolved in methanol. Evaporation of the solvent under reduced pressure gave N-(N-phenylalanylleucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (1.67 g, 95%).

8. N-(N-(N-(N-(1,1-Dimethylethoxycarbonyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide. N-(1,1-Dimethylethoxycarbonyl)glycine 2,4,5-trichlorophenyl ester (1.58 g, 2.55 mmol) (Example B, Intermediate C) and 4-(dimethylamino)pyridine (3.1 g, 2.5 mmol) were added to N-(N-phenylalanylleucyl)glycine N-(2-(N(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (904 mg, 2.55 mmol) and N,N-diisopropylethylamine (990 mg, 7.7 mmol) in dichloromethane (20 mL). The mixture was stirred for 4 d. The solution was washed with cold aqueous sulphuric acid (10%), aqueous sodium carbonate (10%) and saturated brine. The solution was dried with anhydrous magnesium sulphate and the solvent was evaporated under reduced pressure. Chromatography (silica gel; chloroform, then chloroform/methanol 10:1) of the residue gave N-(N-(N-(N-(1,1-dimethylethoxycarbonyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (1.14 g, 61%).

9. N-(N-(N-glycylphenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride. N-(N-(N-(N-(1,1 -Dimethylethoxycarbonyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (1.29 g, 1.77 mmol) was treated with excess hydrogen chloride in dichloromethane (10 mL) for 1 h. Methanol (1 mL) was added and the solvents and excess reagents were evaporated under reduced pressure to give N-(N-(N-glycylphenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (1.1 g, quantitative).

10. N-(N-(N-(N-(N$^{\alpha}$-(1,1-Dimethylethoxycarbonyl)-N$^{\epsilon}$-(2,2,2-trichloroethoxycarbonyl)lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxy-carbonyl)sarcosyl-amino)ethyl)amide. N-(N-(N-Glycylphenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (1.11 g, 1.77 mmol) was added to N,N-diisopropylethylamine (683 mg, 5.3 mmol) in dichloromethane (10 mL). To this mixture was added N$^{\alpha}$-(1,1-dimethylethoxycarbonyl)-N$^{\epsilon}$-(2,2,2-trichloroethoxycarbonyl)lysine 2,4,5-trichlorophenyl ester (950 mg, 1.77 mmol) (Example B, Intermediate D) in dichloromethane (20 mL) and 4-(dimethylamino)pyridine (10 mg). The mixture was stirred for 3 d. The solution was washed with cold aqueous sulphuric acid (10%), aqueous sodium carbonate (10%) and saturated brine. The solution was dried with anhydrous magnesium sulphate and the solvent was evaporated under reduced pressure. Chromatography (silica gel; chloroform, then chloroform/methanol 10:1) of the residue gave N-(N-(N-(N-(N$^{\alpha}$-(1,1-dimethylethoxycarbonyl)-N$^{\epsilon}$-(2,2,2-trichloroethoxycarbonyl)lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide. (1.44 g, 78%).

11. N-(N-(N-(N-(N$^{\epsilon}$-(2,2,2-Trichloroethoxycarbonyl)lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochlo-

ride. N-(N-(N-(N-(N$^{\alpha}$-(1,1-Dimethylethoxycarbonyl)-N$^{\epsilon}$-(2,2,2-trichloroethoxycarbonyl)lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (1.32 g, 1.27 mmol) was treated with excess hydrogen chloride in dichloromethane (20 mL) for 1 h. Methanol (1.0 mL) was added and the mixture was filtered. The solvent was evaporated from the filtrate under reduced pressure to give N-(N-(N-(N-(N$^{\epsilon}$-(2,2,2-trichloroethoxycarbonyl)lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (1.14 g, 92%).

12. N-(N-(N-(N-(N$^{\alpha}$-(N-(Phenylmethoxycarbonyl)sarcosyl)-N$^{\epsilon}$-(2,2,2-trichloroethoxycarbonyl) lysyl)glycyl)phenylalanyl)-leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosyl-amino)ethyl)amide. N-(N-(N-(N-(N$^{\alpha}$-(2,2,2-Trichlorosethoxycarbonyl)lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide hydrochloride (980 mg, 1.0 mmol) was stirred with N,N-diisopropylethylamine (402 mg, 3.1 mmol), N-(phenylmethoxycarbonyl)sarcosine 2,4,5-trichlorophenyl ester (418 mg, 1.0 mmol) (Example B, Intermediate E) and 4-(dimethylamino)pyridine (10 mg) in dichloromethane (30 mL) for 24 h. The solution was washed with saturated aqueous sodium hydrogen carbonate and with aqueous sulphuric acid (2M) and was dried with anhydrous magnesium sulphate. The solvent was evaporated under reduced pressure. Chromatography (silica gel, chloroform/methanol 20:1, then chloroform/methanol 10:1) of the residue gave N-(N-(N-(N-(N$^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)-N$^{\epsilon}$-(2,2,2-trichloroethoxycarbonyl)lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide (418 mg, 36%).

13. N-(N-(N-(N-(N$^{\alpha}$-(N-(Phenylmethoxycarbonyl)sarcosyl)lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide. It is contemplated that N-(N-(N-(N-(N$^{\alpha}$-(N-(Phenylmethoxycarbonyl)sarcosyl)-N$^{\epsilon}$-(2,2,2-trichloroethoxycarbonyl)lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino) ethyl)amide is boiled under reflux with zinc powder in methanol for 2 h. The solvent is evaporated under reduced pressure. Ethyl acetate is added to the residue. The suspension is filtered and the filtrate is washed with twice with water. The solution is dried with anhydrous magnesium sulphate and the solvent is evaporated under reduced pressure to give N-(N-(N-(N-(N$^{\alpha}$-(N-(phenylmethoxycarbonyl)sarcosyl)lysyl)glycyl)phenylalanyl) leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide.

14. N-(N-(N-(N-(N-(N-(Phenylmethoxycarbonyl)sarcosyl)-N-(4-oxo-4-(4-(10,15,20-tripheny-21H,23H-porphin-5-yl)phenylamino)butanoyl)lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide. It is contemplated that 4-oxo-4-(4-(10,15,20-tripheny-21H,23H-porphin-5-yl)phenylamino)butanoic acid (Example B Intermediate E 3) is stirred with pentafluorophenol and dicyclohexylcarbodiimide in dimethylformamide for 16 h at 4° C. The suspension is filtered and the filtrate was added to N-(N-(N-(N-(N-(N(phenylmethoxycarbonyl)sarcosyl)lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide and 4-(dimethylamino) pyridine in tetrahydrofuran. The mixture is stirred for 2 d. Ethyl acetate is added and the solution is washed thrice with water, twice with 10% aqueous sodium carbonate solution and once with saturated brine. The solution is dried with anhydrous magnesium sulphate and the solvent is evaporated under reduced pressure. Chromatography (silica gel) of the residue gives N-(N-(N-(N-(N-(N-(phenylmethoxycarbonyl)sarcosyl)-N-(4-oxo-4-(4-(10,15,20-triphenyl-21H,23H-porphin-5-yl)phenylamino)butanoyl)lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide.

15. N-(N-(N-(N-(N-Sarcosyl-N-(4-oxo-4-(4-(10,15,20-tripheny-21H,23H-porphin-5-yl) phenylamino)butanoyl)lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-sarcosylaminoethyl)amide dihydrobromide. It is contemplated that N-(N-(N-(N-(N-(N-(Phenylmethoxycarbonyl)sarcosyl)-N-(4-oxo-4-(4-(10,15,20-triphenyl-21H,23H-porphin-5-yl)phenylamino)butanoyl)lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-(N-(phenylmethoxycarbonyl)sarcosylamino)ethyl)amide is stirred with 30% hydrogen bromide in acetic acid for 1 h. The solvent and excess reagent is evaporated under reduced pressure. Trituration of the residue with five portions of dry diethyl ether give N-(N-(N-(N-(N-sarcosyl-N-(4-oxo-4-(4-(10,15,20-tripheny-21H,23H-porphin-5-yl)phenylamino)butanoyl) lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-sarcosylaminoethyl)amide dihydrobromide.

16. Polymer B. It is contemplated that N-(N-(N-(N-(N-Sarcosyl-N-(4-oxo-4-(4-(10,15,20-triphenyl-21H,23H-porphin-5-yl)phenylamino)butanoyl)lysyl)glycyl)phenylalanyl)leucyl)glycine N-(2-sarcosylaminoethyl)amide dihydrobromide is boiled under reflux with anhydrous sodium carbonate and poly(oxyethylene)-,-bis(oxiranylmethyl) ether (prepared by the literature method [Y. Chen and M. Feng, Chinese Patent 86 104 089, 1987]) in ethanol for 6 h. The suspension is filtered and the solvent is evaporated from the filtrate under reduced pressure to give the polymer.

Actual levels of active ingredient in administered compositions of the present invention may be varied so as to obtain an amount of active ingredient that is effective to obtain the desired effect for a particular composition and method of administration. The selected dosage level therefore depends upon the desired effect, on the route of administration, on the desired duration of treatment and other commonly considered factors.

The total daily dose of the compounds of this invention administered to a host in single or divided dose may be in amounts, for example, of from about 1 picomol to about 10 millimoles of cytotoxic agent per kilogram of body weight. Dosage unit compositions may contain such amounts or such submultiples thereof as may be used to make up the daily dose. It will be understood, however, that the specific dose level for any particular patient will depend upon a variety of factors including the body weight, general health, sex, diet, time and route of administration, rates of absorption and excretion, combination with other drugs and the severity of the particular disease being treated.

The dosages of the contrast agent used according to the method of the present invention will vary according to the precise nature of the contrast agent used. Preferably however, the dosage should be kept as low as is consistent with achieving contrast enhanced imaging and volumes minimized for IV drip or bolus injection. In this way, the toxicity potential is minimized. For most contrast agents the appropriate dosage will generally range from 0.02 to 3 mmol paramagnetic metal/kg body weight, especially 0.05 to 1.5 mmol/kg, particularly 0.08 to 0.5, more especially 0.1 to 0.4

mmol/kg. It is well within the skill of the average practitioner in this field to determine the optimum dosage for any particular contrast agent for both in vivo or in vitro applications.

Contrast agents may be formulated with conventional pharmaceutical or veterinary aids, for example stabilizers, antioxidants, osmolality adjusting agents, buffers, pH adjusting agents, etc., and may be in a form suitable for injection or infusion directly or after dispersion in or dilution with a physiologically acceptable carrier medium, e.g., water for injection. Thus the contrast agents may be formulated in conventional administration forms such as powders, solutions, suspensions, dispersions, etc., however solutions, suspensions and dispersions in physiologically acceptable carrier media will generally be preferred.

The contrast agents may be formulated for administration using physiologically acceptable carriers or excipients in a manner fully within the skill of the art. For example, the compounds, optionally with the addition of pharmaceutically acceptable excipients, may be suspended or dissolved in an aqueous medium, with the resulting solution or suspension then being sterilized.

Parenterally administrable forms, e.g., intravenous solutions, should of course be sterile and free from physiologically unacceptable agents, and should have low osmolality to minimize irritation or other adverse effects upon administration. Thus, the contrast medium should preferably be isotonic or slightly hypertonic. Suitable vehicles include aqueous vehicles customarily used for administering parenteral solutions such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection and other solutions such as are described in *Remington's Pharmaceutical Sciences*, 15th ed., Easton: Mack Publishing Co., pp. 1405–1412 and 1461–1487 (1975) and *The National Formulary XIV*, 14th ed. Washington: American Pharmaceutical Association (1975). The solutions can contain preservatives, antimicrobial agents, buffers and antioxidants conventionally used for parenteral solutions, excipients and other additives which are compatible with the contrast agents and which will not interfere with the manufacture, storage or use of products.

The present invention includes one or more of the polymers of this invention formulated into compositions together with one or more non-toxic physiologically acceptable carriers, adjuvants or vehicles which are collectively referred to herein as carriers, for parenteral injection, for oral administration in solid or liquid form, for rectal or topical administration, or the like.

The compositions can be administered to humans and animals either orally, rectally, parenterally (intravenous, intramuscularly or subcutaneously), intracisternally, intravaginally, intraperitoneally, locally (powders, ointments or drops), or as a buccal or nasal spray.

Compositions suitable for parenteral injection may comprise physiologically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions and sterile powders and lyophilizates for reconstitution into sterile injectable solutions or dispersions. Examples of suitable aqueous and nonaqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols (propyleneglycol, polyethyleneglycol, glycerol, and the like), suitable mixtures thereof, vegetable oils (such as olive oil) and injectable organic esters such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersions and by the use of surfactants.

These compositions may also contain adjuvants such as preserving, wetting, emulsifying, cryoprotecting, and dispensing agents. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, sorbic acid, and the like. It may also be desirable to include isotonic agents, for example sugars, sodium chloride and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin.

Solid dosage forms for oral administration include capsules, tablets, pills, powders and granules. In such solid dosage forms, the active compound is admixed with at least one inert customary excipient (or carrier) such as sodium citrate or dicalcium phosphate or (a) fillers or extenders, as for example, starches, lactose, sucrose, glucose, mannitol and silicic acid, (b) binders, as for example, carboxymethylcellulose, alignates, gelatin, polyvinylpyrrolidone, sucrose and acacia, (c) humectants, as for example, glycerol, (d) disintegrating agents, as for example, agar, calcium carbonate, potato or tapioca starch, alginic acid, certain complex silicates and sodium carbonate, (e) solution retarders, as for example paraffin, (f) absorption accelerators, as for example, quaternary ammonium compounds, (g) wetting agents, as for example, cetyl alcohol and glycerol monostearate, (h) adsorbents, as for example, kaolin and bentonite, and (i) lubricants, as for example, talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate or mixtures thereof. In the case of capsules, tablets and pills, the dosage forms may also comprise buffering agents.

Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethyleneglycols, and the like.

Solid dosage forms such as tablets, dragees, capsules, pills and granules can be prepared with coatings and shells, such as enteric coatings and others well known in the art. They may contain opacifying agents, and can also be of such composition that they release the active compound or compounds in a certain part of the intestinal tract in a delayed manner. Examples of embedding compositions which can be used are polymeric substances and waxes.

The active compounds can also be in micro-encapsulated form, if appropriate, with one or more of the above-mentioned excipients.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, solutions, suspensions, syrups and elixirs. In addition to the active compounds, the liquid dosage forms may contain inert diluents commonly used in the art, such as water or other solvents, solubilizing agents and emulsifiers, as for example, ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propyleneglycol, 1,3-butyleneglycol, dimethylformamide, oils, in particular, cottonseed oil, groundnut oil, corn germ oil, olive oil, castor oil and sesame oil, glycerol, tetrahydrofurfuryl alcohol, polyethyleneglycols and fatty acid esters of sorbitan or mixtures of these substances, and the like.

Besides such inert diluents, the composition can also include adjuvants, such as wetting agents, emulsifying and suspending agents, sweetening, flavoring and perfuming agents.

Suspensions, in addition to the active compounds, may contain suspending agents, as for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide,

bentonite, agar and tragacanth, or mixtures of these substances, and the like.

Compositions for rectal or vaginal administrations are preferably suppositories which can be prepared by mixing the compounds of the present invention with suitable non-irritating excipients or carriers such as cocoa butter, polyethyleneglycol or a suppository wax, which are solid at ordinary temperatures but liquid at body temperature and therefore, melt in the rectum or vaginal cavity and release the active component.

Dosage forms for topical administration of a compound of this invention include ointments, powders, sprays and inhalants. The active component is admixed under sterile conditions with a physiologically acceptable carrier and any preservatives, buffers or propellants as may be required. Opthalmic formulations, eye ointments, powders and solutions are also contemplated as being within the scope of this invention.

We claim:

1. A linear block copolymer comprising units of an alkylene oxide, linked to units of peptide via a linking group comprising a —$CH_2CHOHCH_2(N)R$— moiety, wherein R is C_{1-4} alkyl group.

2. A linear block copolymer, according to claim 1, wherein the linking group is selected from the group consisting of:

—$CONH(CH_2)_pNHCOCH_2N(CH_3)CH_2CHOHCH_2OC_6H_4$—;

—$CONH(CH_2)_pNHCOCH_2N(CH_3)CH_2CHOHCH_2OC_6H_4CO$—;

—$CONH(CH_2)pNHCOCH_2N(CH_3)CH_2CHOHCH_2OC_6H_4(CH_2)_2$—;

—$CONH(CH_2)_pNHCOCH_2N(CH_3)CH_2CHOHCH_2OC_6H_4(CH_2)_2NH$—;

—$NH(CH_2)_pN(CH_3)CH_2CHOHCH_2OC_6H_4$—;

—$NH(CH_2)_pN(CH_3)CH_2CHOHCH_2OC_6H_4CO$—;

—$NH(CH_2)_pN(CH_3)CH_2CHOHCH_2OC_6H_4(CH_2)_2$—;

—$NH(CH_2)_pN(CH_3)CH_2CHOHCH_2OC_6H_4(CH_2)_2NH$—;

—$CONH(CH_2)_pNHCO(CH_2)pN(CH_3)CH_2CHOHCH_2$—;

—$NH(CH_2)_pNHCO(CH_2)pN(CH_2)CH_2CHOHCH_2$—;

—$NHCO(CH_2)_pN(CH_3)CH_2CHOHCH_2$—; and

—$CO(CH_2)_pN(CH_3)CH_2CHOHCH_2$—

wherein p is 1–6.

3. A copolymer according to claim 2 wherein the peptide is of about 3 to about 50 amino acids in length.

4. A copolymer according to claim 2 wherein the units of alkylene oxide are units of ethylene oxide.

5. A copolymer according to claim 3 wherein the units of alkylene oxide are units of ethylene oxide.

6. A copolymer according to claim 2 wherein the molecular weight is 10,000 to 1 million.

7. A prodrug or drug in a copolymer according to claim 6.

8. A passive targeting copolymer according to claim 6.

9. A copolymer according to claim 6, wherein the peptide is derivatized with a metal chelating agent.

10. A copolymer according to claim 9 wherein the chelating agent has a metal associated therewith.

11. A copolymer according to claim 10 wherein the metal is paramagnetic.

12. A diagnostic imaging copolymer according to claim 11.

13. A copolymer according to claim 10 wherein the metal is a radionuclide.

14. A cytotoxic copolymer according to claim 13.

15. A copolymer according to claim 14 having a molecular weight of 70 kd to 80 kd.

16. A block copolymer according to claim 1 selected from the group consisting of;

—(PAG)$N(R)CH_2CHOHCH_2OC_6H_4CO$(peptide)$NH(CH_2)_pC_6H_4OCH_2CHOHCH_2N(R)$— and

—(PAG)$CH_2CHOHCH_2N(R)CH_2CO$(Peptide)$NH(CH_2)_pNHCOCH_2N(R)CH_2CHOHCH_2$

wherein

R is a 1–4 carbon alkyl group;

p is from 1 to 6;

PAG is polyethylene oxide; and the peptide is Gly-Phe-Leu-Gly; or Lys-Gly-Phe-Leu-Gly.

17. The process of preparing a polymer according to claim 1 by reacting a bis(epoxide) reagent with a bis(amine) reagent, wherein one of said reagents incorporates said peptide units and the other reagent incorporates said alkylene oxide units.

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